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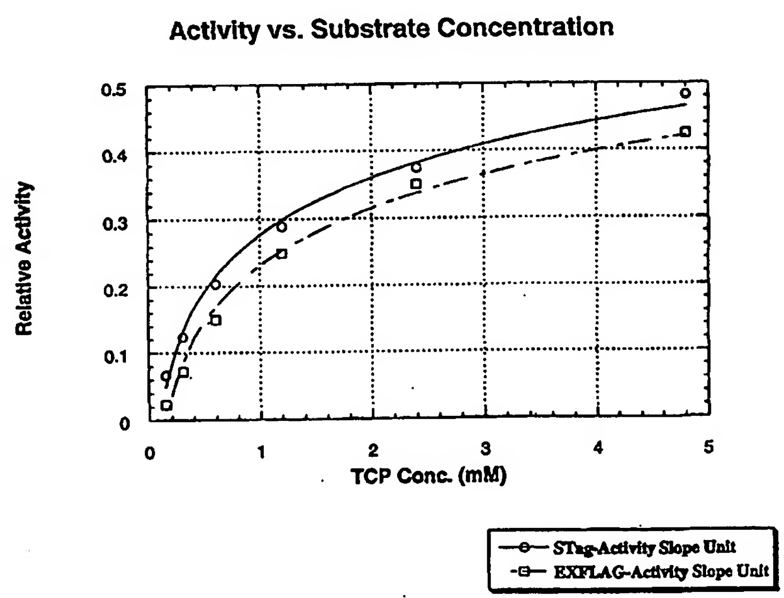

(57) Abstract

The present invention is to haloaliphatic dehalogenase enzymes capable of converting halogenated aliphatic substrate molecules to vicinal halohydrins, as well as to DNA sequences encoding the polypeptide of the enzymes, to expression constructs containing this DNA, and to methods for producing the enzymes by placing the expression constructs into host cells under conditions sufficient for the transformants to produce the dehalogenase. A process for immobilizing the enzyme on a solid support and use of the immobilized enzyme for converting a halogenated aliphatic hydrocarbon to an alcohol is also disclosed.

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RECOMBINANT HALOALIPHATIC DEHALOGENASES

Large quantities of short-chain halogenated aliphatic hydrocarbons (HAHs) are produced for use as organic solvents , degreasing agents, pesticides, intermediates for the synthesis of various other organic compounds and as ingredients in the manufacture of plastics. The extensive use of these halogenated compounds in industrial processes creates a substantial opportunity for new technologies capable of upgrading and/or recycling low-value co-products.

Excess HAHs produced as co-products in chemical manufacturing process can be burned to produce heat and, in some cases, be recycled to low value starting materials, thus yielding some recovery from a waste product or excess co-product. In complex microbial environments (nature, water treatment plants, etc.), HAH degradation occurs by microbial biodegradation. Biodegradation of HAHs results in the formation of carbon dioxide, water, and hydrochloric acid when the halogen is a chloride.

The biodegradation of HAHs to carbon dioxide, water, and hydrochloric acid by select microorganisms is disclosed in U.S. Patent Nos. 4,853,334 and 4,877,736. A process for the decomposition of chlorinated aliphatic hydrocarbons, without specifying the microorganism involved is described in U.S. Patent No. 4,749,491. In addition, the aerobic metabolism of trichloroethylene by *Acinetobacter* spp. has been reported by Nelson *et al.*, *Appl. Environ. Microbiol.*, 52:383-384 (1986). An overview of the degradation of halogenated aliphatic compounds in the environment is given in Vogel *et al.*, *Environ. Sci. Technol.*, 21:722-736 (1987). U. S. Pat. No. 5,372,944 discloses a *Rhodoccocus* species which produces a dehalogenase which converts HAHs to halohydrins. However, these references largely rely on cellular systems and do not take advantage of the benefits that may be obtained from the use of an immobilized, activity-modified enzyme in a continuous feed process. Most relevantly, U.S. Patent No. 5,372,944 relies on *Rhodococcus* cultures comprising wild type or mutant cells. However, the mutation techniques taught therein do not take advantage of recombinant DNA methods and so fail to capitalize on the benefits these methods offer in terms of improvement in activity and expression of the dehalogenase enzyme.

Rather than depend on biodegradation of HAHs by cell cultures, it would be advantageous to have an improved, recombinant enzyme that can be readily adapted to continuous-feed methods whereby the HAHs could be efficiently converted to valuable

intermediates for use in production of other useful products, such as chemical intermediates in the preparation of polyethers to form polyurethanes or in the preparation of glycols and polyglycols to form lubricants, surfactants, emulsifiers, etc.

The present invention is directed to a recombinant enzyme, capable of converting HAHs to vicinal halohydrins, comprising an amino acid sequence substantially homologous with the amino acid sequence of residues 1-292 of Figure 2. Another object of the invention is to provide DNA sequences encoding a polypeptide comprising such an enzyme, more specifically to DNA sequences comprising a polynucleotide substantially homologous with the nucleotide sequence of bases 37-912 of Figure 2.

Another object of the invention is to provide a vector containing the DNA sequence(s) and a method for producing the polypeptide comprising placing the vector into a host cell and growing the host cell under conditions allowing the transformant to produce the dehalogenase.

Further objects of the present invention are to provide an immobilized form of the enzyme on a solid support as well as a process for converting a HAH to an alcohol or halohydrin comprising contacting the HAH with the immobilized enzyme.

Brief Description of Drawings

Figure 1 illustrates a plasmid map of the vector pEXPROK. Plasmid pEXPROK is derived from the commercially available pPROK-1 plasmid (Clontech, Mountain View, CA) containing the Ptac promoter and the 5S, T1T2 terminator sequences. In the figure, the T1T2 region is indicator by "Term." This plasmid was generated by replacing the pPROK-1 multiple cloning site with a pair of oligonucleotides which introduced restriction site *Nco* I, *Hind* III, *Xho* I, *Nhe* I, and *Not* I into the linker. The "ATG" sequence of the Nco I site represents a functional in-frame start site. The Nhe I site is followed by the EXFLAG linker sequence. The sequence of the EXFLAG linker corresponds to nucleotides 919-975 in Figure 2 and encodes amino acids 295-315 in the RDhl protein sequence shown in Figure 2.

Figure 2 (*i.e.* Figures 2A and 2B) presents the nucleotide sequence encoding the putative *Rhodococcus rhodochrous* TDTM003 haloalkane dehalogenase enzyme and the amino acid sequence derived from this nucleotide sequence. Amino acid residues 1-292 correspond to the Rhodococcus dehalogenase (RDhl) structural gene and are encoded by nucleotides 37-912. Amino acid residues -12 through -1 (nucleotides 1-36) represent a polyhistidine-containing amino-terminal tail, with residues -12 and -11 participating in the

formation of both the translational start site and the Nco I cloning site. Amino residues 293-294 (nucleotides 913-918) are encoded by the Nhe I cloning site and are followed by amino acids 295-305, which are referred to herein as the EXFLAG peptide. The EXFLAG linker (nucleotides 919-975) encodes the EXFLAG peptide and a dual-translational stop site (each indicated by an asterisk).

Figure 3 illustrates a plasmid map of the vector pEXPROK-RDhl.

Figure 4 (i.e. Figures 4A and 4B) presents an alignment comparison chart of the amino acid sequences of the putative *Rhodococcus rhodochrous* TDTM003 haloalkane dehalogenase, the *Xanthobacter autotrophicus* GJ10 dehalogenase, the *Renilla reniformis* luciferin monooxygenase, and the *Pseudomonas spp.* LinB gene product (a tetrachloro-cyclohexadiene hydrolase).

Figure 5 presents a plasmid map of the vector pRDhl-KO2.3-EXPROK comprising the putative *Rhodococcus rhodochrous* TDTM003 haloalkane dehalogenase gene under the control of the IPTG-inducible *Ptac* transcription promoter.

Figure 6 illustrates a plasmid map of the high level expression vector pRSET-RDhl comprising the putative *Rhodococcus rhodochrous* TDTM003 haloalkane dehalogenase gene under the control of the T7 transcription promoter.

Figure 7 illustrates a plasmid map of the high level expression vector pTrcHis-RDhl comprising the putative *Rhodococcus rhodochrous* TDTM003 haloalkane dehalogenase gene under the control of the *trc* transcription promoter.

Figure 8 illustrates a plasmid map of the high level expression vector pTrxFus-RDhl comprising a modified version of the putative *Rhodococcus rhodochrous* TDTM003 haloalkane dehalogenase gene fused to the gene encoding *E. coli* thioredoxin, the combined fusion gene being under the control of the P_L transcription promoter.

Figure 9 presents an image of an SDS-PAGE gel of cell lysate samples from cells expressing the pEXPROK-RDhl clone, compared to the partially purified rRDhl enzyme.

Figure 10 presents an image of an anti-FLAG antibody immunoblot of an SDS-PAGE gel identical to that of Figure 9.

Figure 11 presents an image of an SDS-PAGE gel of cell-free extracts from cells expressing pRSET-RDhl.

Figure 12 presents an image of an anti-FLAG antibody immunoblot of an SDS-PAGE gel identical to that of Figure 11.

Figure 13 presents an image of an SDS-PAGE gel of cell-free extracts from cells expressing pTrcHis-RDhl.

Figure 14 presents an image of an anti-FLAG antibody immunoblot of an SDS-PAGE gel identical to that of Figure 13.

Figure 15 presents an image of an SDS-PAGE gel of cell-free extracts from cells expressing pTrxFus-RDhl.

Figure 16 presents a productivity profile for an immobilized enzyme bioreactor acting on the substrate, 1,2,3-Trichloropropane.

Figure 17 presents a bar chart of the activities of EPPCR-mutated *Rhodococcus rhodochrous* haloalkane dehalogenases.

Figure 18 presents a bar chart of the activities of EPPCR-mutated *Rhodococcus rhodochrous* haloalkane dehalogenases.

Figure 19 presents a graph of enzyme activity data for an RDhl enzyme bearing a carboxy-terminal S-Tag polypeptide tail and for an RDhl enzyme bearing a carboxy-terminal EXFLAG polypeptide tail.

The present invention results from intensive research into obtaining a DNA sequence encoding a polypeptide having haloaliphatic dehalogenase activity from a microorganism belonging to the genus *Rhodococcus*, making recombinant DNA sequences by integrating the DNA sequence per se – or as modified – into a vector, and transforming a microorganism with the recombinant vector. Transformants were screened for dehalogenase activity levels and from those with heightened activity, the dehalogenase enzymes were isolated. Various solid support immobilization systems were then evaluated to identify enzyme-support combinations in which the enzyme could effectively convert halogenated aliphatic hydrocarbons to alcohols or halohydrins.

Halogenated aliphatic hydrocarbons (HAHs) subject to conversion using the immobilized dehalogenase include C_2-C_{10} aliphatic hydrocarbon molecules and groups which have two or more halogen atoms attached, wherein at least two of the halogens are on adjacent carbon atoms. Preferred HAHs are saturated hydrocarbons in which at least one of the halogens occupies a primary position on the molecule or group; more preferred are those in which no more than 1 halogen occupies the same carbon atom. Especially preferred HAHs are saturated hydrocarbons comprising 1,2-dihalo groups, examples of which are the 1,2-dihaloethane, 1,2-dihalopropane, 1,2-dihalobutane, and 1,2,3-

trihalopropane molecules and groups. These classes include, for example, 1,2-dichloroethane, 1,2-dichloropropane, 1,2,-dichlorobutane, 1,2,3-trichloropropane, and 1,2-dibromo-3-chloropropane molecules and groups.

As used herein, the term "halogen" means chlorine, bromine, or iodine. The preferred halogens are bromine and chlorine. The most preferred halogen is chlorine and among the most preferred HAHs are volatile chlorinated aliphatic hydrocarbon (VCAH) molecules and groups; especially preferred VCAHs include 1,2-dichloropropane and 1,2,3-trichloropropane molecules and groups.

As used herein, the term "halohydrin" means a vicinal halohydrin, *i.e.* any aliphatic organic compound, other than a carboxylic acid, which contains both a hydroxyl substituent and a halogen substituent on adjacent carbon atoms of the molecule. α,β-halohydrins are the most preferred vicinal halohydrins.

The terms "immunoblot" and "immunoblotting" are used herein to denote the process of: 1) transferring protein(s) from an electrophoresis gel, *e.g.*, a polyacrylamide gel for use in PAGE, to a protein-binding membrane; and then 2) probing that membrane with an antibody specific to protein constituents that may be included among those transferred to the membrane; and then 3) determining the location of that antibody using any of various chromogenic methods well known in the art, *e.g.*, developing color in a colorable marker which is directly or indirectly linked to the antibody. An example of an immunoblotting method is the Western blot.

The terms "permeablize," "permeablizing," and "permeablization" are used herein to denote the process of making something permeable, *e.g.*, to make cell walls permeable. The term "sonicate" is used herein to denote the use of sonic waves to rapidly vibrate the contents of a test tube or other container, in order to thoroughly mix them. The term "vortex" is used herein to denote the action of mechanically gyrating a test tube along its bottom while manually holding the top of the test tube stationary, in order to mix its contents.

The word "selectable" as used herein, means "able to be selected." For example, the phrase "selectable marker" or "dominant selectable marker" indicates a genetic feature, such as a gene encoding an antibiotic resistance enzyme, whose presence allows the gene's host cell to multiply in a corresponding selection medium, *e.g.*, a growth medium containing that antibiotic. When such a genetic feature is incorporated into a plasmid containing a gene encoding a RDhl enzyme, and cells are then treated to receive the plasmid, growing the cells in a selection medium allows the cells actually receiving the plasmid to grow selectively, in

contrast to those cells which did not receive or retain the plasmid. This permits the ready identification of cells which contain the RDhI gene.

As used herein, the phrase "expression construct" denotes a plasmid, virus, virion, viroid, transposable element, *cos*-construct, transfectable carrier-associated DNA strand (*e.g.*, a DNA-coated "gene-gun" pellet or DNA-coated natural or synthetic histone-like particle), or other DNA-to-cell delivery system which is known in the art.

As used herein, in the context of describing amino acid sequences, the following single letter designations apply.

A, a	Alanine (Ala)	M, m	Methionine (Met)
C, c	Cysteine (Cys)	N, n	Asparagine (Asn)
D, d	Aspartic Acid (Asp)	P, p	Proline (Pro)
E, e	Glutamic Acid (Glu)	Q, q	Glutamine (Gln)
F, f	Phenylalanine (Phe)	R, r	Arginine (Arg)
G, g	Glycine (Gly)	S, s	Serine (Ser)
H, h	Histidine (His)	T, t	Threonine (Thr)
I, i	Isoleucine (Ile)	V, v	Valine (Val)
K, k	Lysine (Lys)	W, w	Tryptophan (Trp)
L, l	Leucine (Leu)	Y, y	Tyrosine (Tyr)

As used herein, in the context of describing DNA sequences, the following single letter designations apply:

A	Adenine	G	Guanine	N	A, C, G, or T
C	Cytosine	T	Thymine	R	A or G
				Y	C or T

The following abbreviations and definitions are used herein:

@	At, *e.g.*, @37°C is "at 37°C" and @60min. is "at 60 minutes"
Å	Ångstroms (one Ångstrom is 1×10^{-10} meters)
A	Absorbance, *e.g.*, A_{280} is "absorbance measured at 280nm"
aa	Amino acid
Amp	Ampicillin
2-AMP	2-Aminopropanol
AMPSO	3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid
ATCC	American Type Culture Collection (Rockville, MD, USA)
base	A nucleotide which is part of a polynucleotide
bp	Base pairs
CAPSO	3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid
CD	Compact disc
CHES	2-(N-cyclohexylamino)ethanesulfonic acid
CM	Carboxymethyl
CnBr	Cyanogen bromide
Δ	Change or difference, *e.g.*, ΔA is "change in absorbance"
dATP	Deoxyadenosine triphosphate
DCB	1,4-Dichlorobutane

DCH	2,3-Dichloro-1-propanol
dCTP	Deoxycytidine triphosphate
DEAE	Diethylaminoethyl
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid or ethylenediamine tetraacetate
EPPCR	Error-prone polymerase chain reaction
GC	Gas chromatography
GlA	Glutaraldehyde
gm	Grams
hr	Hours
Hz	Hertz (a measure of frequency in units of cycles per second)
ID	Internal diameter
Ig	Immunoglobulin, *e.g.*, IgG is "immunoglobulin G"
IPTG	Isopropylthiogalactopyranoside
IUB	International Union of Biochemistry
kbp	Kilo-base pairs
kD	Kilo-Daltons (one Dalton weighs $^{1}/12$ of a ^{12}O atom)
K_i	Inhibition constant
LB	Luria broth
µg	Micrograms
µL	Microliters
µM	Micromolar
µmole	Micromoles
M	Molar (moles of solute per liter of solution)
mg	Milligrams
min.	Minutes
mL	Milliliters
mm	Millimeters
mM	Millimolar
MW	Molecular weight
N	Normal (moles of chemically active solute groups per liter of solution, *e.g.*, H_2SO_4 has two acid hydrogens and so 1M H_2SO_4 is a 2N solution)
nm	Nanometers
ng	Nanograms
NP-40	Nonoxynol; p-(n-C_9H_{19})-C_6H_4-$(OCH_2CH_2)_nOH$; also called nonylphenoxypolyethoxyethanol (a non-ionic detergent surfactant)
OD	Optical density, *e.g.*, OD_{600} "optical density measured at 600nm"
oligo	Oligonucleotide
p__	Plasmid, *e.g.*, pRSET, pTrcHis, pTrxFus, or pUC
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
pfu	Plaque forming units
phage	Bacteriophage
QAE	Quaternized ethyl ammonium (an anion exchange group)
RDhl	*Rhodococcus* haloalkane dehalogenase enzyme
residue	An amino acid which is part of a polypeptide
rpm	Rotations per minute
rRDhl	Recombinant *Rhodococcus* haloalkane dehalogenase enzyme
SDS	Sodium dodecyl sulfate
spp.	Species

TCP	1,2,3-Trichloropropane
TM	Trademark
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
U	Units
V_{max}	Maximum enzymatic velocity
% w/v	Percent by weight per volume, *i.e.* number of grams of solute per 100 mL of solution, also written as "% (w/v)"
% w/w	Percent by weight per weight, *i.e.* number of grams of a substance per 100 grams of a mixture containing that substance; also written as "% (w/w)"
~	Approximately

The following steps were carried out in the hope of obtaining an enzyme, and an immobilized enzyme, meeting the objectives of the present invention. These steps were performed using techniques known to those skilled in the art:

(1) isolation and partial determination of the amino acid sequence of a dehalogenase enzyme;

(2) construction of oligonucleotide probes based on the partial sequence determination;

(3) isolation of a dehalogenase-encoding DNA fragment by use of the oligonucleotide probes, followed by amplification the DNA;

(4) ligation of the fragment into a cloning vector having a suitable origin of replication and a gene encoding a dominant selectable marker;

(5) transformation and selection of a microorganism containing the recombinant plasmid;

(6) transference of the DNA sequence to a suitable expression vector and using this recombinant vector to transform a host cell;

(7) production of the recombinant dehalogenase by the transformant; and

(8) purification of the dehalogenase; followed by

(9) immobilization of the dehalogenase onto a variety of solid supports;

(10) use of the immobilized dehalogenase in a process for conversion of HAHs to alcohols or halohydrins; and

(11) selection of effective dehalogenase support systems.

Surprisingly, in the process of performing the above-outlined studies, novel recombinant dehalogenase enzymes were obtained that have performance characteristics

superior to those of the wild-type enzyme from which the recombinant enzymes were derived. In addition, effective immobilized dehalogenase support systems were identified.

The dehalogenase for use in the present invention is preferably derived from *Rhodococcus* species ATCC 55388 and is capable of converting a HAH to a halohydrin or alcohol, preferably a halohydrin. The preferred recombinant enzyme comprises an enzymatically active polypeptide comprising the minimal functional portion of the wild type dehalogenase enzyme, *i.e.* the smallest possible segment thereof which, after proper folding, retains haloalkane dehalogenase activity. Preferably, this polypeptide is substantially homologous with the amino acid sequence of residues 1-292 of Figure 2. More preferably, this polypeptide is at least about 90% homologous, even more preferably at least about 95% homologous, and yet more preferably at least about 99% homologous therewith. Especially preferred are enzymatically active polypeptides having the amino acid sequence of residues 1-292 or of residues of Figure 2.

The preferred recombinant enzyme may also comprise one or more other units such as labels, tags, tails, linkers, solid supports, chelants, other enzymes, and so forth – regardless of their size – which may either be produced with or linked to the enzymatically active polypeptide of the enzyme after it is formed. Such units may be excised from the enzyme after it has been properly folded and/or immobilized upon a solid support. In a preferred embodiment, the enzyme is produced with or linked to a substantially hydrophilic tail. This tail may be a hydrophilic oligopeptide expressed as part of the enzyme or may be, *e.g.*, an oligosaccharide moiety attached by the host cell to the core enzyme after expression thereof. The tail must be of sufficient length and hydrophilicity as to allow the core enzyme to remain in suspension in an aqueous medium. A preferred tail is a substantially hydrophilic oligopeptide expressed as part of the enzyme. More preferably, the enzyme is expressed with a highly hydrophilic oligopeptide tail. Most preferably, the oligopeptide tail is expressed at the carboxy terminus of the enzyme. A most preferred oligopeptide tail is a hydrophilic, carboxy-terminal tail which is rich in histidine and/or aspartic acid residues, especially one which is from about 5 to about 25 amino acids in length and contains at least about 25% histidine or aspartic acid residues, more preferably at least about 50% of such residues. The recombinant enzyme is preferably produced by a host cell containing at least a section of a polynucleotide having the nucleotide sequence of bases 37-912 of Figure 2.

The present invention is also directed to recombinant DNA sequences capable of expressing the enzymes of the present invention. These DNA sequences include those able to express the novel haloalkane dehalogenase(s) by means of translation systems not

following, or not fully following, the standard DNA code's codon-to-amino acid correspondence pattern. Such systems include those in which certain codons are "suppressed" relative to the standard DNA code. In one type of a "suppressed" expression system, at least one of the 20 or so amino-acid-specific classes of aminoacyl-tRNA ("aa-tRNA") molecules contains at least one tRNA molecule – having an anticodon belonging to that class – which is linked to the "wrong" amino acid, so as to predispose the translation system to produce a "violation" of the standard DNA code (*i.e.* by causing the insertion, in at least one position in the growing polypeptide chain, of an amino acid not normally found in correspondence with the mRNA codon governing that position). In another variation on such a system, the pool of amino-acyl-tRNA molecules contains an aa-tRNA whose anticodon is complementary to an mRNA codon normally signaling initiation or termination of translation, thus suppressing the signal. These systems may exist, *e.g.*, as a result of mutations in one or more tRNA molecules or aa-tRNA synthetases, a result of mistakes by non-mutated aa-tRNA synthetase(s), or a result of human intervention in forcing the non-standard linkage of an amino acid to a tRNA.

In such a translation system, a DNA sequence of the present invention will still produce the novel haloalkane dehalogenase(s) either because the insertion(s) of the "wrong" amino acid do not cause the enzyme to lack activity or because the DNA sequence contains – at the position(s) where an "incorrect" amino acid would otherwise be inserted – a codon that "anticipates" the change in the translation system so as to allow either the insertion of the "correct" amino acid or the "correct" signaling of the mRNA codon therein. A preferred DNA sequence comprises a polynucleotide substantially homologous with the nucleotide sequence of bases 37-912 of Figure 2. More preferably, this polynucleotide is at least about 90% homologous, even more preferably at least about 95% homologous, and yet more preferably at least about 99% homologous therewith. Especially preferred are polynucleotides having the amino acid sequence of bases 37-912 of Figure 2.

As used herein, the phrase "substantially homologous" expresses the degree of similarity of a subject sequence – *i.e.* a subject nucleotide sequence (of an oligo- or poly-nucleotide or DNA strand) or a subject amino acid sequence (of an oligo- or poly-peptide or protein) – to a related, reference nucleotide or amino acid sequence. This phrase is defined as at least about 75% "correspondence" (*i.e.* the state of identical elements – nucleotides or amino acids – being situated in parallel) between the subject and reference sequences when those sequences are in "alignment." In this context, "alignment" is said to exist when a minimal number of "null" elements have been inserted in the subject and/or reference

sequences so as to maximize the number of existing elements in correspondence between the sequences. "Null" elements are not part of the subject and reference sequences; also, the minimal number of "null" elements inserted in the subject sequence may differ from the minimal number inserted in the reference sequence. Increased degrees of homology of a given sequence, which may be expressed as, *e.g.*, "90% homologous," are likewise defined with reference to their degree of sequence identicality to a reference sequence.

In this definition, a reference sequence is considered "related" to a subject sequence where either: 1) both nucleotide sequences encode proteins or portions of proteins which may be identified to the same IUB subclass or 2) both amino acid sequences make up proteins or portions of proteins which may be identified to the same IUB subclass, regardless of whether such identification is based on functional properties, sequence homology, or parental origin. "Parental origin" refers to the fact that a given enzyme may initially be grouped within an IUB subclass because of its recognized major or minor function(s), but after the DNA sequence encoding that enzyme accumulates one or more mutation(s), the encoded enzyme may exhibit functional capacities of a different IUB subclass – whether or not the enzyme also retains its original functionality; the "different IUB subclass" may fall within the same or a different IUB main class. The reference to "portions of proteins" signifies that bi- and multi-functional enzymes – including fusion proteins – are also contemplated as falling within a given IUB subclass based on the identification to that subclass of one of their functional domains.

In a preferred embodiment of the present invention, the haloalkane dehalogenase at least parentally belongs to IUB sub-subclass 3.8.1. The enzymes of the present invention have been found to possess unexpectedly superior properties to those of the wild-type haloalkane dehalogenase enzyme found in *Rhodococcus* as, *e.g.*, was utilized in U.S. Patent No. 5,372,944. Generally, aside from its stability under reaction conditions, two characteristics of a given enzyme will determine its usefulness in commercial processes: its affinity for product, as well as its affinity for substrate. Where an enzyme's affinity for product molecules is relatively high, it will be extremely sensitive to feedback inhibition by the product. Such an enzyme will be less useful in commercial processes in which enzymes are often required to operate in the presence of significant product concentrations. A convenient indicator of an enzyme's relative affinity for product is its inhibition constant measured at 90% inhibition ("$K_i(90)$"), *i.e.* the product concentration at which the enzyme retains only 10% of its Vmax, the Vmax being measured when the concentration of product is 0. In regard to the present invention, whereas the wild type haloalkane dehalogenase has a measured

$K_i(90)$ of 20 mM, the recombinant enzyme of the present invention (see Figure 2) has a measured $K_i(90)$ of 50 mM. In other words, the recombinant enzyme is much less sensitive to feedback inhibition by product and can therefore operate in the presence of product concentrations that would essentially shut off the wild type enzyme altogether.

The enzyme of the present invention may be expressed alone, or covalently attached, along its amino and/or carboxy terminus, to one or more polypeptide tail(s). Such tails may be encoded by exons separate from the enzyme-encoding exon or by DNA sequences which are part of the enzyme-encoding exon. When the tail-encoding DNA is to be part of the enzyme-encoding exon, the tail-encoding DNA may be attached or "fused" to the 3' and/or 5' end of the enzyme gene, *e.g.*, either: 1) during enzyme gene amplification by including the tail-encoding nucleotide sequence in an oligonucleotide primer or 2) during plasmid construction by ligating the tail-encoding DNA directly into a plasmid which contains the enzyme gene (whether the enzyme gene is inserted into the plasmid before or after insertion of the tail-encoding DNA).

Under the influence of the appropriate genetic control elements – *i.e.* enhancers, promoters, transcription and translation start and stop sequences, and so forth – expression of such DNA (or mRNA) fusion genes results in production of dehalogenase enzymes with polypeptide tails on one or both ends. An example of a preferred tail-free enzyme is that having the amino acid sequence of residues 1-292 of Figure 2. Examples of some preferred polypeptide tails include poly-histidine sequences, polyacid (*e.g.*, poly-aspartic and/or -glutamic acid) sequences, cellulose binding domains, and the c-myc, S-Tag, and FLAG peptides. Antibodies and affinity columns that bind these exemplary tails are commercially available and may be readily used to purify or immobilize the expressed fusion proteins. However, many other tails may be used while retaining a functional dehalogenase enzyme. Whether or not a tail-encoding sequence is included in the expressed gene, the gene must include, in a position outside the enzyme gene or the enzyme-tail fusion gene, a translation start site, preferably ATG, and will also preferably include an endonuclease restriction site.

In one preferred embodiment, the open reading frame of a single exon encodes a functional dehalogenase enzyme having tails of up to about 30 amino acid residues on the amino and/or carboxy termini. In this embodiment, when both termini have tails, the tails may be of approximately equal length. In another preferred embodiment, the enzyme is expressed with both an amino and a carboxy terminal tail, but the carboxy terminal tail is significantly longer than the amino terminal one. In this embodiment, preferably the amino-terminal tail is up to about 25 amino acids in length and the carboxy-terminal tail is about 2 to

about 150 amino acids in length. In any of these embodiments, preferably, the amino- and/or carboxy-terminal tail will contain a stretch of at least 5 adjacent histidine residues. In an alternate embodiment, the amino terminal tail is about 10-150 amino acids in length and preferably contains or is itself a poly-histidine sequence. In this embodiment, the enzyme may be reversibly immobilized or reversibly inactivated by contact with a surface coated with chelated divalent metal ions, *e.g.*, Mg^{2+} or Ni^{2+}. In this embodiment, the poly-histidine-containing amino-terminal tail may be so long as to partially or totally block access to the enzyme's active site. In an alternate version of this embodiment, the tail may be designed to contain one or more amino acid residues which change the configuration of the tail from that found in a poly-histidine sequence to a bent, recurved, or flexible-joint configuration allowing increased access to the active site of the enzyme.

In a more preferred embodiment, the open reading frame encodes a functional dehalogenase enzyme with an amino terminal tail of about 1 to about 25 amino acids and a carboxy-terminal extension having a polyhistidine sequence, a FLAG peptide sequence (available from KODAK Imaging Systems/VWR, Rochester, NY) and/or an S-Tag peptide sequence. In an especially preferred embodiment, the open reading frame encodes a functional dehalogenase enzyme having: 1) an amino-terminal tail of up to about 10 amino acids and a polyhistidine sequence and 2) a carboxy-terminal tail comprising (*i.e.* containing) the FLAG (see Figure 2) or S-Tag peptide sequence.

The enzymes and/or tails of the above-described dehalogenase enzymes may be modified by use of the techniques of directed evolution, in order to improve their productivity, stability, and/or inhibition profiles. One directed evolution technique uses the gene shuffling method disclosed in U.S. Patent No. 5,605,793 to Stemmer *et al.*, in which a number of similar DNA sequences are fragmented and reassembled in a random fashion to generate highly diverse libraries which can be screened for enzymes with the attributes of interest. Another version of this technology involves use of error-prone gene amplification technologies. A third version of directed evolution employs a combination of these two methodologies. A fourth version of directed evolution is the so-called "staggered extension" process as disclosed in the publication by Zhao *et al.*, in *Nature Biotechnology* (1998) (currently in press). In a preferred embodiment, error-prone gene amplification is used to introduce semi-random mutations into the dehalogenase gene (*e.g.*, Figure 2, residues 1-292) at a rate of about 1-6 point mutations per gene copy per gene amplification reaction, following which the mutant library is introduced into bacteria, induced to express protein, and

screened for activity, preferably in a spatially addressable grid format (such as a 96 well or a 384 well plate).

Effective use of directed evolution to improve an enzyme or enzyme family requires an optimized mutagenesis strategy as well as an expression system and a screening strategy and screening conditions which effectively detect the desired performance attributes of the enzyme. For (non-random) primer-dependent mutagenesis methods (*e.g.*, error-prone gene amplification and defined primer-based recombination), specific protein subdomains can be easily targeted for mutagenesis by primer design and positioning. In a preferred embodiment, primers are used which allow mutagenesis of the entire transcription and translation domain as it occurs within the expression construct. Preferably, primers are directed exclusively to the protein coding region of the expression construct or target DNA (including tails). In a more preferred embodiment, primers are designed in such a way as to target mutagenesis to the dehalogenase enzyme gene while preserving the sequence of the tails. For example, in relation to Figure 2, the dehalogenase enzyme gene may be the sole mutagenesis target when an error-prone gene amplification technique employs both a primer complementary to nucleotides closely preceding nucleotide 36 and a primer complementary to nucleotides closely following nucleotide 912. Likewise, the entire Figure 2 coding region is the mutagenesis target when the primers anneal outside of the region of nucleotides 1-951; the Figure 2 amino tail or carboxy tail, respectively, is targeted when the primers anneal outside of the region of nucleotides 1-36 or 913-951.

The DNA sequence(s) encoding the enzyme or fusion protein of the present invention will preferably be inserted into an expression vector, followed by transfection of the vector into a host cell, and growth of the host cell under conditions in which it expresses the enzyme. A wide variety of recombinant host-vector expression systems for prokaryotic cells are known and may be used in the invention. For example, commercially available vectors such as pKK233-2, pKK388-1, pSE380, pTrcHis (A, B, and C), pRSET (A, B, and C), pProEX-1, and bacteriophages Lambda (gt11), T3, and T7 are all capable of directing expression of heterologous proteins in *Escherichia coli* and other gram-negative prokaryotes. In these expression formats, a variety of strain-appropriate inducible promoters can also be used. In addition, other prokaryotes (such as those of the genus *Bacillus*, *Pseudomonas*, *Actinomyces*, *Bacillus*, or *Rhodococcus*), eukaryotic microorganisms (such as yeast and fungi, *e.g.*, those of the genus *Pichia*, *Saccharomyces*, or *Aspergillus*, *e.g.*, *Pichia pastoris* or *Saccharomyces cerevisiae*), other eukaryotic cells and cell lines (such as Sf21 cells infected with baculouvirus-derived vectors), and even algal cells are capable of producing, in active

form, heterologous proteins of prokaryotic origin; in the event these other cells are utilized in the present invention, appropriate expression vectors would be selected for use therewith. Whereas numerous prokaryotic expression vectors are available publicly and may be used in the present invention, expression of the novel enzymes is exemplified herein with the use of commercially available vectors from the pTrcHis, pRSET and pTrxFus series (available from Invitrogen of San Diego, CA, USA) in conjunction with *E. coli* host cells.

When a directed evolution technique, such as error-prone gene amplification (*e.g.*, error-prone PCR or "EPPCR), is employed, the DNA of the mutant gene pool produced thereby is digested with appropriate restriction enzymes (*i.e.* those endonucleases having restriction sites located external to the mutagenesis target); next, the mutant genes are purified and ligated into prokaryotic expression vectors to form a plasmid library. Competent host cells, *e.g.*, preferably *E. coli* cells, are then transformed with the plasmid library and grown in a suitable medium: in the case of *E. coli*, the cells are plated on agar containing a selective growth medium. The cells may then be diluted to form individual clones, or in the case of prokaryotes such as *E. coli*, they may undergo an initial growth phase, after which the cell colonies are picked individually and transferred to separate containers, *e.g.*, the wells of a 96 well plate, such that each well contains an individual clone of transformed cells. From this library of clones, individual clones can be expanded, induced to express the protein of interest, and screened for the activity of interest.

Screening for the haloaliphatic dehalogenase activity of the novel enzymes is preferably accomplished by detecting the protons or the halide ions released upon hydrolysis of a carbon-halogen covalent bond in a substrate molecule. In a preferred embodiment, the pH change accompanying the proton release serves as a measure of enzyme activity; this pH change is preferably determined using a fluorescent or visible pH indicator which undergoes measurable color change over the functional pH range of the target enzyme. In an alternate method, multiple parallel pH probes may be utilized.

In the activity screening assay, the assayed mixture will contain: 1) whole cells, permeablized cells, cell lysate, or purified enzymes obtained from cells expressing a mutant dehalogenase, preferably from bacterial cells; 2) a substrate; and 3) a low concentration of buffer (typically < 10 mM). When use of permeablized cells is desired, a chemical detergent (*e.g.*, sodium deoxycholate) or a physical freeze/thaw process may be used to make bacterial cells permeable. The substrate will preferably comprise one or more halogenated aliphatic hydrocarbons as discussed above. The buffer may be selected from any known to be effective or found to be effective over the pH range in which the enzyme retains activity.

In some cases, the cell debris itself will be seen to provide sufficient buffering capacity to allow accurate quantitation of activity. Where an added buffer is used, it will preferably have a pKa in the range of about 6 to about 10, although other buffers may be used. Examples of preferred buffers include glycine, 2-AMP, CAPSO, ethanolamine, CHES, borate, serine, and AMPSO; especially preferred is CAPSO and even more preferred is a concentration of about 5mM CAPSO.

The activity screening assay will also require the use of a detection method. In a preferred embodiment, a pH change is detected. Preferably, a pH indicator will be included in the assayed mixture. Any pH indicator having a color change in the pH range in which the enzyme is active may be used. Preferably, the pH indicator will undergo a color change in the range of about pH6 to about pH10, more preferably in the range of about pH7 to about pH9. Examples of preferred visible pH indicators include m-cresol purple, cresol red, phenol red, bromthymol blue, and thymol blue; examples of preferred fluorescent pH indicators include α-naphthol sulfonic acid, 1,4-naphthol sulfonic acid, coumaric acid, 3,6-dioxyphthalic dinitrile, and orcinaurine. In an alternative embodiment, a pH probe may be utilized to detect the pH change. Especially preferred is the use of the visible pH indicator, m-cresol purple, and even more preferred is a concentration of about 50μM m-cresol purple.

In another preferred embodiment, detection is accomplished by measuring the release of halide ions from the substrate by: 1) including in the assayed mixture a halide-sensitive fluorescent dye, such as lucigenin (available from Molecular Probes of Eugene, OR, USA) – lucigenin is quenched upon contact with halide ions and so a decrease in fluorescence in measured therefrom; or 2) utilizing a halide ion responsive probe device, such as a halide-selective electrode.

In a third preferred embodiment, detection of enzyme activity is accomplished using a coupled enzyme system. For example, a coupled enzyme system may be used to detect the production of product molecules: dehalogenation of haloalkanes results in generation of alcohols, and many alcohols are substrates for one or more commercially available alcohol dehydrogenase enzymes (whose activity is measured by disappearance of NADH). Detection of alcohols via coupling to the NADH requirement of the dehydrogenases is well known in the art.

The enzymes of the present invention may be immobilized onto one or more solid support(s). Enzyme immobilization technologies are most conveniently classified into covalent and non-covalent methods. Covalent methods utilize reactive groups present on

certain amino acid side-chains to bond to a polymeric or inorganic support either directly or by using a bifunctional cross-linking agent. The primary advantage of this approach is the robustness of the linkage. Non-covalent immobilization methods are more numerous and range from direct and indirect (*e.g.*, chelate- or chelant-mediated) ionic, adsorptive, or bioaffinity support associations (*e.g.*, biotin-avidin) to gel-entrapment or microencapsulation.

The choice of a particular immobilization technology for a commercial enzyme process is based on a combination of factors. Of primary importance are the cost of the support matrix and its biocompatible linking or coupling chemistries. Next are the recovery of activity upon immobilization and the robustness of the immobilized support under reaction conditions. Unfortunately, since each enzyme is unique, approaches to finding the best system are empirical. However, in conjunction with the enzymes of the present invention, a preferred method of immobilization involves covalently linking the enzyme to the support by means of reactive groups such as epoxides, activated nucleophiles, isourea, and so forth. These reactive groups may be present on the native surface of the support material or the support material may be modified to bear linkers containing such groups. Preferred linkers include those comprising at least one of: dialdehyde, diacid, diamino, diisocyanate, cyanate, and diimide groups; linkers comprising at least one carbodiimide group may also be used, provided that a diamino group is not used in conjunction with a carbodiimide. Among the preferred solid supports are alumina-based supports and silica-based supports; more preferred are polyethyleneimine-impregnated alumina- or silica-based supports. A preferred method of immobilization comprises pre-treating the solid support with glutaraldehyde and then contacting the support with the enzyme.

Once immobilized, the enzyme may be conveniently used to convert its substrate/reactant into product. This conversion can be performed in any suitable medium which does not substantially affect the activity of the dehalogenase. Preferably the enzymatic conversion is done in a aqueous medium containing either a buffering system or one or more pH-control devices.

The halogenated hydrocarbon substrate is generally added to a reaction medium to the saturation point of the substrate, though in some cases, supersaturated substrate mixtures, substrate emulsions, or pure substrate preparations may also be used. Given the saturation point of most halogenated hydrocarbon substrates, the concentration of halogenated hydrocarbon used will generally range from about 0.005% to about 0.5% (w/v). Preferably, the concentration of the halogenated hydrocarbon is from about 0.005% to about 0.25%. More preferred is a concentration of halogenated hydrocarbon from about 0.005% to

about 0.2% in medium. The substrate may be added to the reaction solution initially, as in a batch method, or be added into the liquid stream of a continuous feed process. In such continuous feed processes, the liquid stream may initially contain substrate or the substrate may be first added thereto as the stream is *en route* to the reactor. In either case, more substrate may be added directly to the liquid stream in the reactor in order to ensure that a high concentration of substrate is presented to the enzyme throughout the reactor. The liquid stream may be re-saturated with substrate at various intervals in the process in order to enable accumulation of product at concentrations higher than the solubility limits of the substrate. The batch method reaction is usually carried out with shaking or stirring. Although the reaction time or reactor residence time may vary depending on the reaction conditions, such as the substrate concentration or the amount of enzyme, the reaction conditions are preferably selected so that the reaction is completed within a maximum of 120 hours.

The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the present invention. All percents are percent by weight unless otherwise indicated.

General Experimental

Materials and Media:

All oligonucleotides were synthesized and purified by Genosys Biotechnologies Inc. (Woodland, TX), Life Technologies, Inc. (Rockville, MD) or Integrated DNA Technologies, Inc. (Coralville, IA). Restriction enzymes and DNA modifying enzymes were purchased from Gibco-Bethesda Research Laboratories (Gaithersburg, MD), New England Biolab Inc. (Beverly, MA), or Stratagene Cloning Systems (La Jolla, CA) and were used according to manufacturer's protocols. Competent *E. coli* AG1 cells were purchased from Stratagene Cloning Systems, Competent *E. coli* JM109 cells and TOP 10F' cells were purchased from Invitrogen Corp. (San Diego, CA). Small scale plasmid DNA isolations were done using the Rapid Pure Miniprep (RPM™) system (BIO 101, Inc., La Jolla, CA). DNA ligations were performed with pre-tested reagent kits purchased from Stratagene Cloning Systems. Purification of DNA fragments was with either QIAquick Gel Extraction Kits and QIAquick PCR Purification Kits both purchased from Qiagen Inc. (Chatsworth, CA). SDS-polyacrylamide gels and associated buffers and stains, as well as electroblot transfer buffers, came from Integrated Separation System (ISS, Natick, MA). Antibodies, anti-

FLAG™ monoclonal antibody M2, and goat anti-mouse IgG1 were obtained from International Biotechnology Inc. (IBI, New Haven, CT) and Southern Biotechnology Associates (Birmingham, AL),.respectively. Bacteria were cultured in Luria-Broth ("LB") using premixed reagents purchased from Gibco-Bethesda Research Laboratories (G-BRL; Gaithersburg, MD).

Reagents

1,4-Dichlorobutane, 60% perchloric acid, ferric nitrate, and mercuric thiocyanate were from Aldrich. Anhydrous ethanol was from Quantum/USI (Tuscola, IL, USA). 1,2,3-Trichloropropane was a gift from The Dow Chemical Company's Allylics Group (Freeport, TX, USA). Monobasic potassium phosphate, dibasic potassium phosphate, imidazole, guanidine hydrochloride, disodium EDTA, ammonium sulfate, and Tris free base were Fisher Biotech Grade. Sulfuric acid was from Fisher (ACS grade).

Support Materials

The Tresyl-Toyopearl chromatography support was from TosoHaas (Lot # 65TRM72R). Sephadex G-25 prepackaged columns were from Pharmacia. Celite R-648 was from Manville. Polyethyleneimine, 50,000 MW and PEI-silica were from Sigma. Glutaraldehyde, Grade 1, as 25% aqueous solution, also from Sigma, was stored at -20°C until just prior to use. Other samples used in immobilization include: Davison Low SA Alumina, Norton SA 6176 Alumina, Calcicat Type C Alumina, Calcicat s-88-473 Type A Silica, Shell 5980-F Silica, Davison 952-08-5X Silica, Borecker subunit Carbon, and AmCy 5701-Sn Carbon.

Methods:

PCR Reactions

DNA Amplification was performed using standard polymerase chain reaction buffers supplied by Perkin-Elmer-Cetus (Nutley, NJ). Typically, 50 µL reactions include 1× concentration of manufacturer supplied buffer, 1.5 mM $MgCl_2$, 125 µM dATP, 125 µM dCTP, 125 µM dGTP, 125 µM dTTP, 0.1-1.0 µM forward and reverse primers, 5U AmpliTaq DNA Polymerase and <1 ng target DNA. Unless otherwise indicated, thermal profile for

amplification of DNA is for 35 cycles of a thermal profile of 0.5 min. @94°C; 1 min. @55°C; 1 min. @72°C.

Protein Detection by Polyacrylamide Gel Electrophoresis

Soluble protein was mixed 1:1 with solubilization buffer (Tris/SDS/ß-mercaptoethanol, pH 6.8; ISS) and boiled for five minutes before being loaded on 10-20% gels (Daiichi, Natick, MA) and electrophoresed with Tris-glycine buffer (ISS). Gels were stained with Pro-Blue™ (ISS).

Standard Chloride Detection Assay to Determine Units of Enzyme Activity

When using 1,4-dichlorobutane (Aldrich) as a substrate, 100 mM NaGlycinate pH 9 was added to each 9 mL capped vial to a final volume of 6 mL. When using 1,2,3-trichloropropane as a substrate, 10 mM TrisSulfate/1 mM EDTA (pH 7.0) was used. Six µL substrate were then added and the contents were vortexed. Vials were incubated at 30°C for 1 hour with stirring. Sampling occurred at 5 time points by removing 1 mL of mixture and placing it in an Eppendorf tube containing 100 µL 0.375 M $Fe^{3+}(NO_3)_3$ in 5.25 M $HClO_4$. Tubes were vortexed. When all samples had been collected, 100 µL mercuric(II) thiocyanate saturated in ethanol was added to each of the tubes. Once again, samples were vortexed, then centrifuged for 3 minutes. Optical densities were read at 460nm. Slopes representing change in absorbance over time (ΔA/min) were determined and divided by 1.52 (the extinction coefficient at 460nm using NaCl as standard in units of ΔA/µmole Cl^-) to give µmole Cl^-/min. One unit of enzyme activity is defined as the amount required to dehalogenate 1.0 µmole of substrate/minute under the specified conditions.

Procedure for Error-Prone PCR Mutagenesis

In this directed evolution procedure, an RDhl enzyme gene or RDhl fusion protein gene was provided as an EPPCR mutagenesis target, *e.g.*, by using appropriate restriction enzymes to digest a plasmid containing the target DNA sequence. In most cases, the target DNA was purified by gel electrophoresis, followed by gel extraction of the target DNA. EPPCR involved performing a standard PCR gene amplification of the target gene, using appropriate oligonucleotide primers, except that the standard PCR buffer was supplemented with sufficient magnesium chloride and manganese chloride to bring the reaction mixture to 7 mM magnesium chloride and 0.15 mM manganese chloride. This procedure may be repeated upon one or more of the EPPCR products to introduce further mutations therein.

The resulting EPPCR products were ligated into expression vectors (*e.g.*, pTrcHis, pTrxFus) and the vectors were then used to transform appropriate, competent host cells, *e.g.*, *E. coli* AG1 or JM109 cells, for enzyme expression and enzyme activity analysis. Plasmid-containing clones were identified by selective growth on LB/Amp agar plates. Individual colonies were transferred by toothpick into the wells of a 96-well plate containing a selective growth medium and incubated at 37°C for ~8-12hr to allow for growth. Following the initial growth phase, replica plates were generated, expanded, and individual clones thereof were assayed for dehalogenase activity as described in the following section.

Procedure for Measuring RDhl Enzyme Activity by Detection of pH Change

RDhl enzyme activity was measured by detecting the pH change resulting from action of the enzyme in dehalogenating substrate. Prokaryotic host cells expressing the enzyme were grown in broth, quantitated, and permeablized prior to addition of a pH indicator, buffer, and substrate.

Each well of a 96-well microplate received 200µL of an SOB broth (obtained from Difco, Detroit, MI, USA) which had been supplemented with about 50-100µg/mL of ampicillin ("SOB/Amp"). Cells from a single colony of enzyme-producing *E. coli* clone were inoculated into one well of the plate. When testing a library of rRDhl enzymes or rRDHL fusion proteins, each well was inoculated with cells from a different *E. coli* clone. Six wells received no cells, in order to serve as a negative control, and six additional wells were inoculated with an *E. coli* clone producing the wild-type RDhl enzyme, as a positive control. The inoculates were incubated overnight in a Psycrotherm oven at 37°C while being shaken at 250 rpm.

After incubation, the cultures were induced by addition of IPTG to a final concentration of 1mM, followed by another 5 hours of incubation at 37°C in a Psycrotherm oven with 150 rpm shaking. After the 5 hour incubation, the cell density of each culture was determined by use of a 1.573 Vmax/Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). 20µL aliquots of each of the induced cultures were then transferred the wells of a fresh 96-well plate and 2.2µL of pH8.0, 10x permeablization buffer (10 mM sodium deoxycholate, 1% NP-40, 50 mM Tris, and 50 mM EDTA) was added to each aliquot, followed by shaking for 3-5 min. at moderate shaking speed. Each of the cell culture aliquots then received 200µL of a DCB-saturated buffer (>1µL DCB/mL buffer system), at pH 9.2-9.5, which contained 5mM CAPSO and 100µM cresol purple. The developing color change of the indicator was measured by use of a SpectraMaxPlus microplate reader

(Molecular Devices, Sunnyvale, CA, USA) and the slope of the color change was plotted to extrapolate the initial enzyme activity.

Example 1

Isolation of dehalogenase enzyme from *Rhodococcus*.

Rhodococcus species ATCC 55388 was cultured as described in U.S. Patent No. 5,372,944. An enzyme extract was prepared from this culture as generally described in U.S. Patent No. 5,373,944 by taking a 25-75% ammonium sulfate cut, two ion exchange chromatography steps (1. DEAE-Sephadex; 2. DEAE-Sephacryl) in which the salt concentration was varied over the range of 0-400 millimolar sodium sulfate in the form of a gradient, gel filtration chromatography using Sephadex G-75, and then concentration by ultrafiltration to obtain an enzyme preparation containing greater than 65% dehalogenase by SDS-polyacrylamide analysis.

A portion (~25 mg) of the purified enzyme was subjected to cyanogen bromide digestion. Peptide fragments were isolated using an RP-8 Macrosphere (Altech) mixed mode cation column with a 0-80% acetonitrile/water gradient containing 0.1% trifluoracetic acid.

Three purified protein and purified cyanogen bromide (CnBr) fragments were subjected to sequencing by automated Edman degradation. The sequences of the N-terminus and three CnBr fragments were determined. One of the CnBr fragments was identical to the N-terminus in sequence. The other two corresponded to unique internal dehalogenase sequences. Sequences of all the peptides are shown in Table 1.

Table 1: Sequences of N-terminal and Proteolytic Fragments Derived from Purified *Rhodococcus* Dehalogenase.

N Terminal Sequence:
SEIGT GFPFD PHYVE VLGER
Cyanogen Bromide Fragment Sequences:
1. HYVDV GPRDG
2. DHYRE PFLKP VDRE

DNA Primer Design

Primers RDhl 5.4 and RDhl 3.12 were designed to allow amplification and cloning of the open reading frame encoding the *Rhodococcus* dehalogenase (RDhl) gene in expression system pEXPROK. The sequence of RDhl 5.4 was derived from the N-terminal sequence of

the protein whereas RDhl 3.12 was designed based on the actual DNA sequence. Primers RDhl 5.7 and RDhl 3.13 were designed to generate an RDhl gene in expression system pRSET and pTrcHis. Primers Trx2++ and Trx- were designed to generate an RDhl gene in expression system pTrxFus.

The sequences of these oligonucleotide primers are as follows:

Table 2: Sequences and Orientation of Oligonucleotide Primers Used in Cloning of the *Rhodococcus* Dehalogenase

Oligo Name	Orientation	Design based on	Sequence*
RDhl 5.4	Forward	N-terminal/homology	5'GGTTCCATGGGNTT(CT)CCNTT(CT)GA(CT)CCNCA(TC)TA
RDhl 3.12	Reverse	3'-Sequence Data	5'Bio-CAGAGCTAGC GAGTCCGGGGAGCCAGCG
RDhl 5.7	Forward		5'CGTACATATGGCCATGGGG GGT TCT CAT CAT CAT Nde 1 Nco 1 G G S H H H CAT CAT CAT GGT ATG TCT GAA ATA GGT ACC H H H GGT TTT CCC TTC GAC CCT CAT TA-3'
RDhl 3.13	Reverse		5'-GAT GAC AAA TAA TGA GCG GCC GCA AGC TTG TAC-3' Not 1 Hind III
Trx2++			5'-CC GGG GAT CCC ATG GCT TCT GAA ATA GGT ACC GGT BamH I Nco I TTT CCC TTC GAC CCT CAT TA-3'
Trx-			5'-TCG ACT GCA GGC GGC CGC TCA TTA TTT GTC ATC-3' Pst I Not I

*Bio=Biotin; N=A, C, G, or T; ( )=defined base redundancy at a given position. Underlined sequences correspond to 5' sequences intended to introduce, into the amplified DNA products, restriction sites compatible with the intended cloning vector (pEXPROK).

Cloning of the Partial *Rhodococcus* Dehalogenase Genes

Cloning of the *Rhodococcus* dehalogenase gene was accomplished by amplification from a genomic DNA library as follows. Genomic DNA was isolated from the *Rhodococcus* ATCC strain 5538 using the methods of P.J. Asturias and K. Timmis (*J. Bacteriology* 175:4631-4640 (1993)). Purified genomic DNA (100 µg) was sheared mechanically to an average size of <10 kbp. Fragments were ligated to *Bam*H I linkers, followed by *Bam*H I digestion and ligation into a *Bam*H I digested preparation of bacteriophage Lambda-ZAP Express™ DNA (obtained from Stratagene, Inc. of LaJolla, CA, USA). A library containing the genomic *Rhodococcus* DNA fragments was prepared commercially (Stratagene, Inc., LaJolla, CA) and supplied at a titer of $1x10^4$ pfu/µL (plaque forming units per microliter). A

redundant DNA primer (RDhl 5.4) corresponding to the codons for amino acids 6-13 of the N-terminal sequence was synthesized using solid phase phosphoramidite chemistry and purified by HPLC (Table 2).

The RDhl 5.4 primer was used in combination with a commercially available primer which recognizes the T3 bacteriophage promoter sequence (and is contained within the Lambda ZAP Express™ vector) to amplify dehalogenase sequences from the singly-expanded genomic DNA bacteriophage library. Amplification was accomplished using the polymerase chain reaction (50 μL) containing 1 μM of RDhl 5.4 primer, 100nM biotinylated T3 Pro primer (New England Biolabs), 10× Amplitaq reaction buffer (Perkin-Elmer-Cetus), 1.5 mM $MgCl_2$, 5U of rAmpliTaq DNA polymerase (Perkin-Elmer-Cetus), and 4 μL of the phage library (whole phage). Amplification was for 35 cycles of the following thermal profile: 1 min. @94°C; 2 min. @ 55°C; 2 min. @72°C. PCR products were separated by electrophoresis through 1.0% agarose and a discrete band of 1.3 kbp was identified, excised from the gel, and isolated using a QiaQuick gel purification kit (Qiagen, Inc.). After confirming that this DNA was also capable of being amplified by other *Rhodococcus* dehalogenase-specific primers, the fragment was digested with restriction enzymes *Nco* I and *Pst* I and ligated into *Nco* I/*Pst* I digested pGEM5zf(+) (ProMega, Madison, WI). Sequencing of the 3′-untranslated region of the cloned segment allowed identification of a putative stop codon and subsequent amplification of the coding region with primers RDhl 5.4 and RDhl 3.12.

Sequence and Restriction Enzyme Analyses. Double stranded sequencing of the dehalogenase gene proceeded via successive rounds of the dideoxy method with the biotinylated primers (Table 3) designed for each successive round, based on the sequencing results in preceding rounds. Bands were separated on 5.5-6.0% polyacrylamide urea sequencing gels, the DNA transferred to nitrocellulose filters by capillary transfer and visualized using the well-known streptavidin-alkaline phosphatase development protocols in combination with chemiluminescent substrates.

Table 3: Sequences and Orientation of Oligonucleotide Primers Used in Sequencing the *Rhodococcus* Dehalogenase Gene

Oligo Name	Orientation	Specific for bp	Sequence*
Dhl Seq 7	Forward	697-714	5'Bio-CCTGTCCCGAAGTTGTTG
Dhl Seq 8	Reverse	807-791	5'Bio-CGGGCCGATGTCCACTG
Dhl Seq 11	Forward	186-202	5'Bio-TGCTCCAGACCTGATCG
Dhl Seq 12	Reverse	496-480	5'Bio-TCTGATCGATGATCAAC
Dhl Seq 13	Forward	404-422	5'Bio-TCCCGACGTGGACGAATG
Dhl Seq 14	Reverse	663-646	5'Bio-GAGCGCGACGATGTTCGC
Dhl Seq 15	Forward	725-742	5'Bio-CACCCGGCGTACTGATCC
Dhl Seq 18	Reverse	951-934	5'Bio-GAGACCGGTCAGCATTCC
PROK-SEQ1	Forward	PROMOTER	5'Bio-GAGCGGATAACAATTTCA
PROK-SEQ2	Reverse	TERMINATOR	5'Bio-TCTCATCCGCCAAAACAG

*Bio=Biotin; N=A, C, G, or T; ( )=defined base redundancy at a given position. Does not include the biotinylated primers already described in Table 2 which also were used to determine the sequence of the gene. Commercially available (New England Biolabs) biotinylated primers specific for the T3, T7, and SP6 promoters were also used but are not listed here.

The vector pEXPROK (Figure 1) is a derivative of the commercially available pPROK-1 vector (Clontech, Inc., Mountain View, CA). Whereas the pEXPROK retains the functional elements of the pPROK vector (including the ampicillin resistance marker, the *Ptac* transcriptional promoter, and paired transcription termination signals following the polylinker), pEXPROK vector replaces the *Eco*R I-to- *Hin*d III polylinker of pPROK-1 with an extended synthetic polylinker referred to as EXFLAG. The EXFLAG linker is designed to allow insertion of an open reading frame between an *Nco* I site and an *Nhe* I site. In-frame with the six-nucleotide *Nhe* I site is an 11 amino acid peptide, the final octapeptide of which corresponds to the well-known FLAG peptide (Kodak Imaging Systems, Rochester, NY) to which antibodies and affinity reagents are commercially available. Sequence and features of the EXFLAG linker are as follows:

EcoR I	Nco I	Hind III	Xba I	Xho I	Nhe I	I-----EXFLAG-
GAATTCAG	CCATGGCAT	AAGCTT	TCTAGA	CTCGAGGGA	GCTAGC	GGC CTA GGT
						Gly Leu Gly

peptide-----------------------→ Not I.

GAC TAC AAG GAC GAT GAT GAC AAA TAA TGA GCGGCCGC TAGCTT

Asp Tyr Lys Asp Asp Asp Asp Lys *** ***

PCR amplification of the RDhl 5.4/T3Pro gene from the pGEM5 construct with primers RDhl 5.4 and RDhl 3.12, followed by digestion with *Nco* I and *Nhe* I, allows ligation of the *Rhodococcus* dehalogenase gene into the appropriately digested expression vector. This procedure was used to insert the RDhl gene into pEXPROK. The plasmid maps of pEXPROK and pEXPROK-RDhl are shown in Figures 1 and 3, respectively. The DNA sequence of the pEXPROK-RDhl construct was later confirmed by automated DNA sequencing.

Sequence Analysis. The complete DNA and derived protein sequences for the dehalogenase gene are shown in Figure 2. DNA Sequence data reveals an open reading frame of 876 bp, giving a deduced protein sequence of 292 amino acids and a predicted molecular weight of 33kD. This is similar to the molecular weight reported for a number of other hydrolytic dehalogenases.

To determine whether the isolated gene is likely to encode a dehalogenating enzyme, a MacVector v.4.5.2 (Kodak, Inc.) sequence analysis package was used to compare the derived protein sequence with those of all other known proteins contained in the Entrez Sequence Database (the Entrez Database is maintained by the National Center for Biotechnology Information). The RDhl polypeptide displays the greatest similarity to members of the so-called α/β hydrolase family of enzymes including several haloalkane and haloacid dehalogenases, epoxide hydroalses, and enzymes with a number of diverse catalytic functions. Alignment of the Dow *Rhodococcus* dehalogenase with two other dehalogenases and a non-dehalogenase (luciferin monooxygenase) enzyme is shown in Figure 4. The enzymes included in the figure and their publication references are as follows: *Xanthobacter autotrophicus* haloalkane dehalogenase – D.B. Janssen, *et al.*, *J. Bacteriology* 171:6791-6799 (1989); tetrachlorocyclohexadiene hydrolase (TCCH or LinB) – Y. Nagata, *et al.*, *J. Bacteriology* 175(20):6403-6410 (1993); *Renilla reniformis* luciferin monooxygenase – W.W. Lorenz, *et al. Proc. National Acad. of Sciences, U.S.A.* 88(10):4438-4442 (1992). More recent releases of the Entrez database also reveal significant similarity between the

Dow RDhl protein and two hypothetical mycobacterium tuberculosis proteins of unknown function (Entrez Database Accession numbers 1449324 and 1478233, submitted 7-22-96 and 7-23-96, respectively, by K. Badcock and C.M. Churcher, *et al.*), as well as with the haloalkane dehalogenase isolated from *Rhodococcus rhodochrous* (Entrez Database Accession number 1196824, submitted 2-15-96 by A.N. Kulakova, *et al.*).

Of the sequences aligned in Figure 4, only the *Xanthobacter* dehalogenase has been well characterized at a structural and mechanistic level. Notably, two of the three residues known to be involved directly in the *Xanthobacter* catalytic cycle (the two most important residues, Asp-124 and His-289) are conserved in the *Rhodococcus* sequence. These similarities and those indicated in the Figure suggest a high degree of structural and mechanistic conservation among members of this family of proteins.

Dehalogenase Protein Expression.

To confirm the identity of the above, cloned enzyme as a dehalogenase, we sought to express the full-length protein in *E. coli.* To accomplish this, a 1300 bp *Nco* I/*Spe* I restriction fragment, containing the RDhl gene was excised from the pRDhlKO2.1-pGEM5 construct and ligated with the *Nco* I/*Nhe* I-digested pEXPROK vector. Because *Spe* I and *Nhe* 1 generate ligation-compatible restriction fragments, this resulted in the generation of an expression construct (Figure 5) containing the complete putative RDhl gene under the transcriptional control of the IPTG-inducible *Ptac* promoter and the termination control of the endogenous RDhl 3′ untranslated region.

Colonies transformed with the resulting plasmid (pRDhlKO2.3-EXPROK) were grown overnight in 2 mL minicultures, following which 1 mL of each culture was pelleted, washed, and sonicated. Extracts were then assayed for their capacity to catalyze release of chloride following addition of the RDhl substrate, 1-chlorobutane. Chloride releasing activity was absent from cultures not containing the cloned gene; cultures with the cloned gene exhibited chloride releasing activity which increased when transcriptional activity of the gene was increased by the addition of IPTG. Thus, dehalogenase activity could be induced in overnight cultures of the recombinant *E. coli* containing the pRDhlKO2.3-pEXPROK construct.

Example 2

The gene encoding this dehalogenating enzyme has been isolated and cloned into the bacterium, *E. coli.* DNA sequence analysis revealed that this isolated gene encodes a

protein with a high degree of sequence similarity to other known dehalogenating enzymes. In an effort to increase levels of biosynthesis to commercially meaningful levels (*i.e.* "expression"), a number of systems reported to enable high level production of heterologous proteins in *E coli* were examined.

To generate the expression vector pEXPROK-RDhl, plasmid pEXPROK was digested with restriction enzymes *Nco* I/*Nhe* I and then purified by a QIAquick Gel Extraction Kit (Qiagen, Inc., Chatsworth, CA). The RDhl open reading frame was amplified with primer RDHL 5.4 as the forward primer (containing an *Nco* I site to direct the start of translation) and primer RDHL 3.12 as the reverse primer (and containing an *Nhe* I site). Following digestion of the amplified DNA with *Nco* I and *Nhe* I, the gene was ligated into the pEXPROK vector. The new construct was then transformed into *E. coli* AG1 competent cells and ampicillin resistant colonies were picked. Plasmids containing the RDhl gene were identified by analytical restriction enzyme digestion and referred to as pEXPROK-RDhl construct. The pEXPROK-RDhl plasmid map is shown in Figure 3.

<u>Construction of pRSET-RDhl and pTrcHis-RDhl Expression Vectors</u>

For construction of both pRSET-RDhl and pTrcHis-RDhl expression vectors, the RDhl gene was amplified from the pEXPROK-RDhl using oligonucleotide primers RDhl 5.4 and RDhl 3.13 using standard PCR conditions. Amplification products were separated on agarose gels and purified using standard procedures.

Both pRSET and pTrcHis vectors are IPTG inducible expression vectors, derived from the pUC 18 and 19 series of cloning vectors. They both were purchased from Invitrogen Corp. (San Diego, CA) and contain the following features:

(a) Both are designed for high level protein expression and both carry an ampicillin resistance gene.

(b) Both contain a sequence that encodes an N-terminal fusion peptide which codes for six histidine residues. These residues function as a metal binding domain and may allow later purification of recombinant protein by affinity chromatography.

(c) The vectors encode an enterokinase cleavage recognition sequence (the FLAG and/or EXFLAG peptide) downstream of the dehalogenase coding region which allows detection by and immobilization upon anti-FLAG antibodies.

The high level expression property of the pRSET vector results from the presence of the T7 promoter upstream of the heterologous gene. Since *E. coli* does not contain the T7

polymerase, however, an M13 phage containing the T7 RNA polymerase gene is needed for protein expression. In practice, bacteria containing a heterologous gene under the control of a T7 promoter are induced to produce the heterologous protein by infection of recombinant *E. coli* with T7 phage containing the T7 RNA polymerase. Alternatively, commercially available E. coli stably expressing the T7 RNA polymerase enzyme (i.e. BL21) can be transformed with the pRSET construct.

The pRSET-RDhI expression vector was generated by digesting plasmid pRSET with restriction enzymes *Nco* I/*Hin*d III and then incorporating an RDhI gene fragment which contains an *Nco* I site at the 5′ end and a *Hin*d III site at the 3′ end. The new construct was then transfected into *E. coli* JM109 competent cells and ampicillin resistant colonies were picked. Plasmids containing the RDhI gene were identified by analytical restriction enzyme digestion and referred to as the pRSET-RDhI construct. The pRSET-RDhI expression construct is shown in Figure 6. One such clone (Clone 16-4) was used to characterize protein expression using the pRSET system.

The pTrcHis vector contains another high level transcriptional promoter – the *trc* promoter, a fusion of the well-characterized *trp* promoter and the *lac* promoter. The pTrcHis vector also contains a mini-cistron upstream of the heterologous gene which provides highly efficient, repeat initiation of translation of the cloned protein in the multiple cloning site.

Using a similar process, we cloned an RDhI gene fragment into the pTrcHis vector to generate the pTrcHis-RDhI expression vector. For expression studies, *E. coli* TOP 10′ competent cells were transformed with the pTrcHis new construct. Both the pRSET-RDhI and pTrcHis-RDhI expression vectors contain an 11 amino acid EXFLAG peptide downstream of the *Nhe* I site.

The EXFLAG peptide sequence is in-frame with the open reading frame of the cloned protein and is useful for analytical detection and affinity purification. Figure 7 shows a map of the completed pTrcHis-RDhI expression construct. One such clone (Clone 18-3) was identified as a high expressing clone and used for further characterization of the TrcHis expression system.

Construction of pTrxFus-RDhI Expression Vector

The ThioFusion™ expression system (Invitrogen Corp., San Diego, CA) provides a means of expressing large amounts of heterologous protein by fusing the gene encoding such a protein to the gene encoding the *E. coli* protein, thioredoxin, in the pTrxFus expression vector. The thioredoxin moiety can confer both solubility and heat stability to its

fusion partner, thereby opening up new options for purification by osmotic shock or heat treatment. The expression vector, pTrxFus, allows foreign genes to be inserted into its multiple cloning site. It uses the P_L promoter from bacteriophage lambda to drive expression and the cI repressor, also from bacteriophage lambda, to control the level of transcription. Expression of the cI repressor gene is under control of the *trp* promoter and repressor. Expression of a foreign gene is induced by adding tryptophan to the medium which shuts down cI repressor synthesis and allows transcription from the P_L promoter.

Primers Trx2++ and Trx- (see DNA Primer Design) were designed to modify the RDhI gene fragment with an enzyme restriction site unique to the TrxFus multiple cloning site. Plasmid pEXPROK-RDhI was used as a template, and a gene fragment was generated by PCR, using primers Trx2++ and Trx-, which added a *Bam*H I site to the 5′ end and a *Pst* I site to the 3′ end. The fragment was purified using a QIAquick PCR Purification Kit. Both the pTrxFus vector and the gene fragment were enzyme-digested, agarose gel purified, and ligated. The new construct, pTrxFus-RDhI (Figure 8), was incorporated into GI174 electrocompetent cells (Invitrogen Corp.) which had been prepared following the manufacturer's instructions.

Expression Analysis

Growth and Induction of Cell Cultures--For expression studies, clones identified as containing proper DNA constructs were cultured in 3 mL of Luria Broth (LB) or SOB medium (Difco, Detroit, MI, USA) containing 50 µg/mL ampicillin in 15 mL round-bottom polypropylene culture tubes. These culture tubes were incubated overnight at 37°C with shaking (200 cycles/minute in a rotary shaker) or grown to an OD_{600} of 0.6. Afterward, 2 mL of fresh medium with IPTG was added (to a final IPTG concentration of 1mM) and the tubes were incubated at 37°C with constant shaking for another 4-5 hours. For recombinant clones of pRSET, after 1 hour of IPTG induction the cell cultures were infected with previously titered M13/T7 phage and the incubation continued as described previously.

For recombinant clones of pTrxFus, RDhI gene-containing clones were cultured in 1 mL RM medium with 100 µg/mL ampicillin and incubated overnight at 30°C with shaking (200 cycles/minute in a rotary shaker). The next day, 9 mL fresh Induction Medium were added and growth continued at 30°C to an OD_{550} of 0.5. Then, cell cultures were induced with tryptophan (to a final concentration of 100 µg/mL) and transferred to a 37°C incubator and shaken at 200 rpm for another 2 to 4 hours.

Cell Free Extract Preparation--For protein analysis, induced, overnight cell cultures were pelleted by centrifugation at 4°C (5000 rpm for 10 minutes in a Sorvall SS-34 rotor). Cell pellets were washed in cold 10 mM Tris sulfate buffer (pH 7.5) containing 1mM disodium EDTA and then centrifuged again. For clones of pEXPROK, pRSET, and pTrcHis, final suspensions were sonicated at 14 Hz on ice through 3 repetitions of a 20 seconds "on", 30 seconds "off" regimen, using a small-tip sonicating probe (Soniprep 150, MSE Ltd., Crawley, Sussex). Insoluble debris was removed by centrifugation at 10,000 rpm for 10 minutes. Cell-free supernatants were then transferred to clean polypropylene tubes and appropriate assays performed. Final cell suspensions from clones of pTrxFus were sonicated for three 10-second bursts and then flash-frozen in a dry ice/ethanol bath. Shortly after freezing, the cell lysates were quickly thawed at 37°C and two more, rapid sonication-freeze-thaw cycles were performed. After the last thaw, the procedures described above for removing the cellular and insoluble debris were continued.

Expression and Purification of pEXPROK-RDhl

Figure 9 shows a Pro-Blue™ stained SDS-PAGE gel of cell lysate samples of the pEXPROK-RDhl clone 12-4 on the left side (lanes 2-5) and partially purified rRDhl enzyme on the right side (lanes 8-11). Lane 1 contains molecular weight standards and lanes 6 and 7 contain single, 60 ng and 180 ng bands of the FLAG-peptide protein at a molecular weight of 55kD. Lanes 2-5 show all the soluble protein from cell-free extracts. Since rRDhl enzyme is not a major protein in the extracts, immunoblotting of an identical gel was done to confirm the presence of this recombinant enzyme. Figure 10 shows this recombinant enzyme band in each sample lane, as recognized by an anti-FLAG antibody at the predicted molecular weight of ~ 35kD. Lanes 6 and 7 in Figure 10 are 20 ng and 60 ng, respectively, of the FLAG-peptide protein. Affinity purified recombinant enzyme was analyzed on both a Pro-Blue™-stained SDS-PAGE gel and an immunoblotting membrane. Four consecutive fractions of affinity-purified rRDhl enzyme were run in lanes 8-11 of the Pro-Blue™-stained SDS-PAGE gel shown in Figure 10. In addition to a prominent band at ~35kD molecular weight, other protein bands are visible on the gel. The immunoblot of the partially purified enzymes (Figure 10, lanes 8-11), however, confirms that the recombinant enzyme at ~35kD is the only protein to stain with anti-FLAG antibodies and thus appears to be the proper translated rRDhl protein. This data suggests that rRDhl enzyme is stable both in the *E. coli* intracellular environment and throughout the purification process.

Expression of pRSET-RDhI and pTrcHis-RDhI

Cell free extracts obtained from clones of the pRSET recombinant enzyme expression system and the pTrcHis recombinant enzyme expression system were analyzed for the presence of recombinant RDhI protein. Five clones containing the correct size *Nco* I/*Hin*d III DNA fragment were identified, cultured overnight, lysed, and analyzed for rRDhI expression by SDS-PAGE gel (Figure 11). Lane 1 shows molecular weight standards and lane 7 and 8 contain single 60 ng & 180 ng bands of the FLAG-peptide protein at a molecular weight of 55kD. Lanes 2-6 show samples of 1 µL of cell-free extracts from the 5 clones and lanes 9-12 show samples of 0.1 µL of the cell-free extracts. Immunoblots of these extracts reveal doublet bands (35kD and 38kD) in each sample lane, when the anti-FLAG antibody is used to stain the immunoblots (Figure 12). This may suggest that there are two start codons in the pRSET-RDhI system. The first start codon was originally designed in the pRSET vector system to be about 41 amino acids (123 bp) before the actual cloning site, which allows the initiation of translation from that Met ATG codon followed by 6 histidines. The second start codon may occur at the *Nco* I cloning site itself, which was designed into the original 5′ end primer of the RDhI gene fragment. However, the presence of an anti-FLAG antibody-reactive band confirms the presence of rRDhI enzyme.

Figure 13 shows the Pro-Blue™-stained SDS-PAGE gel with cell-free extracts from the pTrcHis system and Figure 14 shows the immunoblot of an identical SDS-PAGE gel. All clones of the pTrcHis system show an overloaded, anti-FLAG-reacted band at a molecular weight of ~35 kD, which confirmed the presence of rRDhI enzyme in the extracts. Since the volumes of the initial culture and the cell-free extract preparations are the same in all three systems, these overloaded bands are an indication of higher enzyme production in the pTrcHis system.

Expression of pTrxFus-RDhI

The soluble protein, cell-free extracts from the pTrxFus system were examined for the presence of rRDhI enzyme, using reducing SDS-PAGE. Figure 15 shows a gel stained with Pro-Blue™. Lane 12 shows molecular weight standards and lane 11 shows a single 150ng band of the FLAG-peptide protein at a molecular weight of 55kD. The thioredoxin fusion bands are clearly visible as the major protein in lanes 1 to 9 at a molecular weight of 47 kD. This size corresponds to the 12 kD of the thioredoxin protein and 35 kD of the rRDhI enzyme. In contrast, lane 10 has a sample of insoluble matter from cell lysis, which shows no presence of the high-level, expressed thioredoxin fusion protein. This demonstrates that

all of the fusion protein is in a soluble state. Data from other experiments indicate this fusion protein band can be recognized by anti-FLAG antibody at the same molecular weight in the immunoblot membrane (data not shown).

Analysis of Hydrolytic Dehalogenation Activity

To quantify the hydrolytic dechlorination activity of the recombinant enzyme, a colorimetric chloride-release assay at 460 nm was used.

Recombinant protein activity was measured by adding an appropriate amount of cell-free extract (prepared as described above) to 6.0 mL of reaction buffer in a glass vial. 100 mM sodium glycinate buffer (pH 9.0) was used for measuring activity toward 1,4-dichlorobutane (DCB) (Aldrich Chemical Co.), and 100 mM Tris-SO_4 buffer (pH 7.0) was used for measuring activity toward 1,2,3-trichloropropane (TCP). The halogenated substrate (6μL) and a micro stir bar were added and the vial was capped. Capped vials were incubated in a 30°C water bath with stirring.

Periodically, 1.0 mL samples were removed and assayed for free chloride. Reagent 1, 0.375M Ferric Nitrate in 5.25 N Perchloric acid (10% v/v), was added to stop the hydrolytic reaction and reagent 2, saturated Mercuric Thiocyanate in ethanol (10% v/v), was added to develop color. Final samples were read in a Perkin-Elmer 552A UV/VIS Spectrophotometer at 460 nm. Rates were determined after correcting for non-enzymatic hydrolysis against a blank.

Dehalogenating Activity of Recombinant *Rhodococcus* dehalogenase

While the preceding data suggest that the recombinant dehalogenase can be synthesized at much higher levels in *E. coli* than in wild type *Rhodococcus*, they do not address the activity of the expressed protein. Indeed, enhancing production of a dechlorinating enzyme is the key objective of this work. For this reason, we examined the relative levels of dehalogenase activity in representative clones from each of the above constructs. Activity was determined by the free chloride release assay and compared with protein expression as documented in Figures 13-15. Protein expression was quantified on SDS-PAGE gels by high resolution scanning densitometry and the measured amount of rRDhl was stated in terms of % of total soluble protein. The following table shows the relationship between dehalogenating activity and the percent of rRDhl enzyme in the total soluble protein among all four expression systems.

**

Expression System	% of Total Soluble Protein	DCB* Activity per mL of Culture	Clone Name
pEXPROK	~3	0.3×10^{-2}	EXPROK-RDhl
pRSET	~10	0.8×10^{-2}	RSET RDhl Clone 16-4
pTrcHis	~15	2.4×10^{-2}	TrcHis RDhl Clone 18-3
pTrxFus	~30	4.8×10^{-2}	TrxFus RDhl Clone 4

* DCB units were measured as the degree of indicator color change (ΔOD/min.) as the enzyme dechlorinated 1,4-dichlorobutane.

This data reveals a strong correlation between level of rRDhl protein expression and observed dehalogenase activity.

In this example, *Rhodococcus* haloalkane dehalogenase can be expressed at high levels in *E. Coli* in 3 of the 4 systems examined. The recombinant *Rhodococcus* dehalogenase is stably expressed in all four systems and recognized by anti-FLAG antibodies at the expected molecular weight. This recombinant enzyme exhibits a dehalogenase activity at a level similar to that of the wild type and proportional to the level of heterologous protein expression.

Example 3.

Preparation of Porous Alumina Supports

Protein (385 mg) representing 22 TCP units of activity were immobilized on 2.0 g of volatile-free alumina (lot #1587 of k-4 alumina from UOP of DesPlaines, IL, USA) at 4°C with mild agitation over the weekend. The procedure followed UOP's standard practice of GIA-activation of the polyethyleneimine coating, water washing, and enzyme addition. The bathing solution was decanted and the support was washed five times with 2 mM Tris/1 mM EDTA, pH 7.5.

Enzyme Purification and Preparation for Immobilization

Recombinant *Rhodococcus* dehalogenase was produced in *E. coli* using the pTrcHis expression system. Enzyme preparations used for all immobilization studies were first partially purified using ammonium sulfate precipitation, using a cut of 45 to 70% saturation at 4 °C, followed by dialysis and clarification in 10 mM Tris sulfate, 1 mM EDTA, pH 7.5. This basic buffer was used throughout all purification steps. These preparations were routinely 4.5-fold purified from the lysate, as determined by absorbance at 280 nm, and were

estimated to be 30-35% pure dehalogenase protein by SDS-PAGE. More highly purified enzyme preparations were achieved by an additional DEAE-Sepharose chromatographic step of eluting with a 0-400 mM ammonium sulfate gradient. This provided about 10-fold purification from lysate, with 85-90% enzyme purity. This step was followed by QAE-Sepharose FF chromatography with a narrower 0-120 mM ammonium sulfate gradient, achieving about 12-fold purification from lysate, and SDS-PAGE which demonstrated enzyme homogeneity. Purified RDhl from the TrcHis RDhl expression system is typically referred to herein as "rRDhl."

Preparation of Supports

All anion exchange supports were thoroughly hydrated according to manufacturer's instructions (if necessary), then exhaustively washed to remove ethanol and to exchange into the sulfate form by continuous rinsing with10 mM Tris sulfate, 1 mM EDTA. This same starting buffer was used throughout to load enzyme preparations.

Inorganic supports were modified with polyethyleneimine and glutaraldehyde according to well established protocols (U.S. Patent No. 4,268,410 and Mosbach, *Immobilized Enzymes*, in 44 Methods in Enzymology, (1976) (Academic Press, NY)). Dry samples of supports were weighed out and distributed into 12 mL capped vials. An aqueous solution of 2.5 % polyethyleneimine was added to a total of 10 mL per gram of support. Vials were capped, and then agitated gently on a rocking shaker for 1 hour at room temperature. Samples were transferred to a small Büchner funnel where liquid was removed by gentle vacuum. Supports were transferred to a watch glass and allowed to air dry at room temperature overnight (about 18 hr). Samples were transferred to a new vial to which was added a freshly thawed solution of 25% aqueous glutaraldehyde at a ratio of 20 mL per gm of support. The mixture was capped and shaken intermittently for 1 hour in a hood. The glutaraldehyde was removed by decantation and washed exhaustively with water until no aldehyde was detected by a fuchsin test. Prior to enzyme immobilization, supports were decanted, but not dried.

Immobilization of Enzyme

All enzyme immobilization was performed in a cold room at 4°C using a rocking shaker to provide gentle agitation. Times used for binding of enzyme preparations to supports ranged from 1 hour for the ion-exchange supports to a maximum of 4 days for experiments with the PEI-GIA modified inorganic supports. Buffer exchange was used only for the Celite R 648 binding capacity studies in which the Tris buffer was exchanged for a 10

mM potassium phosphate, 1 mM EDTA, pH 7.0 buffer on a pre-packed Sephadex G-25 column.

Enzyme Stripping from Ion-exchange Supports

Two sets of 250 µL aliquots of resin slurry were bound overnight with either 11.1 DCB U (Toyopearl®) or 6.63 DCB U (PEI Cellulose) of enzyme. The binding supernatants were carefully removed and assayed. Resins were spun at 6,000 rpm for 8 minutes. Additional supernatant was removed and the resins were washed twice with 100 mM Na Glycinate buffer (pH 9). One tube from each set was treated with 0.5 M $(NH_4)_2SO_4$ for 1 hour to strip the enzyme from the support. These resins were rinsed again with buffer. Resins and supernatants were assayed for activity using DCB as a substrate.

Ion-Exchange Supports

Anion-exchange chromatography has been used extensively in the purification and characterization of both the wild type and the recombinant dehalogenase enzymes. The enzyme is anionic at neutral pH (where the dehalogenation reaction is performed) and anion exchange supports often function well in immobilizing such proteins. This approach was also attractive because it allowed for simultaneous purification and immobilization of the enzyme. To confirm this potential utility, we examined binding and elution of the rRDhl protein to anion and cation exchange resins over a wide pH range. Figure 2 shows the nearly quantitative retention of the dehalogenase on DEAE Sepharose anion exchange resin over a range of 5 pH units. For contrast, the CM-Sepharose rapidly loses its binding capacity above pH 5.

Immobilizations

A number of support materials were examined for their efficacy in immobilizing rRDhl. In these studies, a 40-70% ammonium sulfate cut of the dehalogenase enzyme was used. Duplicate sets of enzyme, immobilized on each of thirteen ion-exchange supports were prepared. One of each set was assayed immediately for dehalogenase activity using the chloride release assay. The second was treated with TCP-saturated 10 mM Tris sulfate (pH 7.5) at 45°C for 1 hour. Following this treatment, supernatant was removed and this set was also assayed by the standard chloride method. Table 4 summarizes the results of these assays.

Table 4 Screen of TrcHis RDhl on 13 ion-exchange supports following incubation at 45°C for 1 hour in the presence of substrate.

Support	Supplier	Lot/Batch #	% Activity after TCP Treatment
Silica Gel PEI-Silica	Sigma	24H0810	0
DEAE Sephadex A-50	Sigma	24H0485	19
PEI Cellulose(med. mesh)	Sigma	94H7200	54
Glass, Aminopropyl	Sigma	34H8260	43
Toyopearl® Super Q-650M	TosoHaas	65QAM02RM	79
DEAETrisacryl Plus-M	Sigma	92H0861	21
Spectra/Gel Ion Exchange 1X8	Spectrum	16865	14
Dowex® 1X8-200 Ion Exchange Resin	Aldrich	12627-85-9	54*
DE52	Whatman	1152032	50
Quaternaryammonium Cellulose	Whatman	9852032	2
DEAE Sepharose	Sigma	53H0177	30
AG 3X4 100-200	Bio-Rad	52594A	18
AG 4X4 100-200	Bio-Rad	47426A	9

*incubated at 37°C

These results indicate that there is marked heterogeneity in the efficacy of these matrices as supports for the dehalogenase enzymes. Similar heterogeneity will be seen for similar dehalogenases catalyzing similar reactions.

Four of the best candidates were screened for stability over time in the presence of TCP. These were: PEI cellulose, Toyopearl® Super Q-650M, Glass Aminopropyl, and DEAE Sepharose. Duplicate sets were prepared, one to be used for an initial chloride detection assay, the second for assay after exposure to TCP. Table 5 shows that 3 of the 4 lost significant activity in the first 24 hours but retained stable activity out to at least 7 days following the initial loss. Toyopearl® underwent a similar but delayed loss at 48-120 hours and then appeared to stabilize.

Table 5: Stability Study of TrcHis RDHL on 4 Ion-Exchange Supports in Presence of TCP at Room Temperature

Support	% Activity Over Time			
	24 hr	48 hr	120 hr	192 hr
PEI Cellulose(med. mesh) Sigma	83	79	78	78
Glass, Aminopropyl Sigma	78	87	75	66
Toyopearl® Super Q-650M TosoHaas	100	100	78	82
DEAE Sepharose Sigma	76	79	73	62

All four resins appear to be good candidates for immobilizing the dehalogenase and appear to provide a suitable surface for prolonged enzyme activity.

Covalent Coupling to Tresyl-Activated Polyacrylic Polymer

In order to determine the feasibility of covalently coupling rRDhl through pendant amino groups to any support, an activated resin, Tresyl-Toyopearl, was evaluated. This activated resin provides a stable secondary amino group linkage with the enzyme:

$$\text{)-O-R-O-}CH_2\text{-}CH_2OS\overset{\overset{O}{\|}}{O}CH_2CF_3 + H_2N\text{-Enzyme} \rightarrow \text{)-O-R-O-}CH_2\text{-}CH_2\text{-NH-Enzyme}$$

The rRDhl preparation used for these studies had been affinity purified from *E. coli* lysate using the anti-FLAG antibody column, and was estimated to be about 20% pure by SDS-PAGE. 0.35 units of enzyme (1.96 mg total protein) were coupled to 40 mg of the Tresyl-Toyopearl under conditions described by the manufacturer. After 3 hours, 93% of protein had been coupled as determined by the decrease observed at A_{280}. An additional 10 mg of resin were added and coupling continued for 1 hour to bind >98% of the protein. Re-assay of the washed gel for dehalogenase activity revealed recovery of 0.11 units of activity (31%). A second trial using 2.6 units of the same enzyme preparation and 1.0 gm of activated resin demonstrated a recovery of 37% activity. According to manufacturer's notes, recoveries of activity from enzymes coupled to this support usually lie in the 40-60% range, so 31-37% represents reasonable recovery and is sufficient to make commercially practical the coupling of the enzyme, via its amino groups, to an immobilization support material for use in a bench-size or industrial bioreactor.

This covalent attachment to hydrophilic resins is also an effective means of immobilizing the dehalogenase enzyme.

Polyethyleneimine Impregnated Inorganic Supports Cross-Linked with Glutaraldehyde

Inorganic supports have also found wide utility in the industrial enzyme arena due to availability, low cost, high loading capacity, ease of regeneration and reuse, and the wide range of pore sizes. Porous alumina, silica, and Celite have found widespread use as supports for immobilized enzymes, with titanium- and carbon-based supports seeing more limited application.

Enzymes can be immobilized to inorganic supports by three mechanisms. The enzyme may associate with the inorganic support through ionic interactions or may bind through an ion-exchange mechanism to an ionic polymer which has been impregnated into the inorganic support, or be crosslinked to the ionic polymer using a bifunctional chemical linker. The first approach has not seen wide applicability because the weak ionic interactions frequently lead to enzyme leaching. Polyethyleneimine (PEI) is the polymer of choice for impregnation because of low cost. The amino groups allow a wide range of crosslinking chemistry to be applied. Glutaraldehyde is by far the most studied and inexpensive crosslinking agent used. Studies with rRDhl focused entirely on this coupling chemistry.

Established methods for the preparation of PEI-impregnated porous supports, followed by glutaraldehyde crosslinking were used (U.S. Patent No. 4,268,410 and Mosbach, *Immobilized Enzymes*, in 44 Methods in Enzymology, (1976) (Academic Press, NY)). The recovery of rRDhl activity was initially screened for two supports. Porous silica already impregnated with PEI was obtained from Sigma (nominal pore size of 250 Å). Celite R-648 was obtained from Manville (nominal pore size of about 150 Å) and impregnated with PEI (avg. 50,000 MW) from Sigma according to the methods of U.S. Patent No. 4,268,410. Both supports were treated with glutaraldehyde (GlA) and then washed exhaustively with water. 5.5 units (1.0 mL at 0.55 mg protein/mL) of a highly purified rRDhl enzyme preparation (>98% pure by SDS-PAGE) was used to couple to 500 mg each of the two glutaraldehyde-treated, PEI-impregnated supports. This loading level (0.11% w/w) was assumed to be at least two orders of magnitude below the known loading capacity of the supports. The enzyme was incubated with the supports, with gentle shaking overnight (18 hr) at 4 °C, before washing exhaustively to remove unlinked protein. Re-assay of the two supports with DCB demonstrated recoveries of 40% for the PEI-Celite R-648 and 31% for the PEI-Silica.

These samples were stored at room temperature under reaction conditions (saturating DCB) for 1 week and re-assayed. The Celite immobilized enzyme preparation lost 49% of activity in a week while the Silica immobilized enzyme preparation lost 28% of its activity.

In order to quickly determine which type of inorganic support would provide the best recovery of rRDhl activity, several commercially available porous supports were screened. As in the previous experiment, loading levels of the highly purified rRDhl enzyme were set greater than three orders of magnitude (0.0055 % w/w) below the expected loading capacities of the supports in order to compare the supports on the sole basis of activity recovered, independent of loading capacity, pore size and so forth.

Three porous aluminas, three porous silicas, and two porous carbons were screened. Additionally, Sigma PEI-Silica and Celite R-648 (evaluated in the previous screen) were reevaluated under the same conditions. All supports were impregnated with PEI and treated with glutaraldehyde as before. 25 µL of enzyme preparation (13.8 µg) were incubated with each support for 72 hours at 4 °C with gentle agitation to ensure maximum loading. Enzyme loading in the bathing solution was monitored by measurement of A_{280} at 24 and 72 hours. Activity against DCB was then determined in the bathing solution (unbound) and on the washed gels (bound) after the 72 hour incubation. Tables 6 and 7 show the results of these studies.

Table 6 rRDhl Enzyme Uptake into PEI-impregnated GIA treated Porous Supports as Monitored by A280

Support	%Loaded @ 24 hr	% Loaded @ 72 hr
Alumina - Davison Low SA	83%	62%
Alumina - Norton SA 6176	86%	84%
Alumina - Calcicat Type C	84%	67%
Silica - Calcicat S-88-473 TypeA	69%	72%
Silica - Shell 5980-F	81%	93%
Silica - Davison 952-08-5X	91%	92%
Carbon - Borecker Subunit	77%	91%
Carbon - AmCy 5701-Sn	90%	95%
Celite - Manville R648	82%	95%
PEI-Silica - Sigma	85%	93%

Table 7 Recovery of Enzyme Activity on PEI-Impregnated GIA treated Supports

Support	% Bound	% Unbound	%Lost*
Alumina - Davison Low SA	7%	38%	55%
Alumina - Norton SA 6176	3%	17%	80%
Alumina - Calcicat Type C	7%	34%	59%
Silica - Calcicat S-88-473 Type A	12%	28%	60%
Silica - Shell 5980-F	12%	8%	80%
Silica - Davison 952-08-5X	17%	11%	72%
Carbon - Borecker Subunit	5%	9%	86%
Carbon - AmCy 5701-Sn	7%	5%	88%
Celite - Manville R648	21%	5%	74%
PEI-Silica - Sigma	6%	7%	87%

*% Lost = 100% - (% Bound + % Unbound)

Each support exhibits a different uptake profile ranging from 62% to 95% uptake after 72 hours. For most systems, 72 hours is adequate to achieve maximum loading of protein achievable at 4 °C. However, the three alumina systems actually showed greater uptake at 24 hours than at 72 hours. Recovery of bound enzyme activities at 72 hours ranged from 3% to 21% as compared to an untreated soluble enzyme control. Considerable activity was unaccounted for or "lost" in all systems examined, ranging from 55% to 87%. Also, the previously run supports (Sigma PEI-Silica and Manville Celite R648) showed poorer bound recoveries. This could be due to either the lower enzyme loading ratio or the longer incubation times used in this experiment. Given the efficiency of binding and the recovery of bound enzyme activity, Celite, silica, carbon, and alumina all function as effective immobilization support materials in the present invention, although Celite and silicas outperform alumina and carbon. In terms of stability, however, alumina supports appear to perform better (see below).

The bound samples were also submitted to a long term stability study. Following assay using DCB as a substrate, supports were rinsed and immersed in TCP-saturated buffer. At the given time-point, TCP buffer was removed, supports were rinsed again and assayed with DCB.

Table 8 Long term stability study of PEI cross-linked supports at room temperature.

Support	% Activity Maintained at 41 hr	% Activity Maintained at 136 hr
Alumina 1	38	57
Alumina 2	66	67
Alumina 3	58	75
Silica 1	76	81
Silica 2	79	46
Silica 3	60	30
Carbon 1	75	0
Carbon 2	37	48
Celite	57	12
PEI-Silica	43	60

Thus the two supports which exhibited intermediate levels of recovery in the immobilization reaction, silicas and aluminas, proved to have the best stabilities over time. All of these supports were also screened for their ability to bind the enzyme directly without PEI or GIA modification. However, binding was very poor and irreproducible, and allowed easy removal of enzyme from the supports with washing.

Polyethyleneimine-Impregnated Inorganic Supports with Enzyme Bound by Ion Exchange

The molecular weight of PEI is also known to have an impact on the overall yield and stability of immobilized enzymes. In addition, PEI is capable of functioning either as an ion exchange ligand on various supports or as a glutaraldehyde cross-link acceptor. For these reasons, PEIs of two different molecular weights were impregnated onto various porous inorganic supports following the method described in the previous section. In these experiments, however, enzyme (semi-purified preparations containing 1-2 U/mL) was bound by ion-exchange but the GIA crosslinking step was omitted. Samples were submitted for a stability screen to determine if the size of the PEI was an important factor.

Table 9 Stability Screen of PEI-Impregnated Porous Supports, No Crosslinking

	Support	Supplier	MW PEI	% Activity Maintained at 24 hr	at 120 hr
1	Alumina	Norton SA 6176	50,000	70	62
2	Alumina	Calcicat Type C	"	61	42
3	Silica	Calcicat S-88-473 Type A	"	101	64
4	Silica	Shell 5980-F	"	80	55
5	Carbon	Borecker Subunit	"	41	32
6	Carbon	AmCy 5701-Sn	"	39	17
7	Celite	Manville R648	"	83	50
8	Alumina	Norton SA 6176	2000	82	55
9	Alumina	Calcicat Type C	"	91	61
10	Silica	Calcicat S-88-473 Type A	"	94	57
11	Silica	Shell 5980-F	"	76	55
12	Carbon	Borecker Subunit	"	44	50
13	Carbon	AmCy 5701-Sn	"	42	21
14	Celite	Manville R648	"	58	2

With the exception of Celite, the different molecular weights of PEI did not appear to have a major impact on either immobilization efficiency or stability of the enzyme.

Example 4

Construction of pRSET-RDhl.Nde

The pRSET-RDhl.Nde expression vector was generated by digesting plasmid pRSET RDhl clone 16-4 with the restriction enzymes, *Nde* I and *Hind* III, and then incorporating into the construct a RDhl gene fragment which contained a *Nde* I site at its 5' end and a *Hind* III site at its 3' end. The new construct was then transformed into *E. coli* JM109 competent cells (from Invitrogen of Carlsbad, CA, USA) and ampicillin resistant colonies were picked. Plasmids containing the RDhl gene were identified by analytical restriction enzyme digestion and referred to as the pRSET-RDhl.Nde construct.

Production of Recombinant RDhl Protein

Both of the new constructs – pRSET-RDhl.Nde and pTrcHis-RDhl – were transformed into *E. coli* B834(DE3) competent cells (from Novagen, Inc. of Madison, WI, USA). The production of active dehalogenase enzymes was confirmed by a dehalogenation activity assay and enzyme production levels were investigated with PAGE. Dehalogenation activity was measured by using a colorimetric chloride release assay at 460 nm to assess enzymatic dechlorination activity toward 1,4-dichlorobutane (DCB).

We observed the enhanced production of recombinant RDhl enzyme in this host-*E. coli* B834(DE3) competent cell. The following table shows the relationship between dehalogenating activity and the percent of rRDhl enzyme in the total soluble protein among different expression systems and host cells.

Expression System	Competent Host Cell	rRDhl as % of Soluble Protein	DCB* Activity per mL of Culture (x 10^{-2})
pEXPROK	E. coli AG 1‡	~3	~0.3
pRSET	E. coli JM 109	~10	~0.8
pTrcHis	E. coli TOP 10F'‡	~15	~2.4
pTrxFus	E. coli GI 174‡	~30	~4.8
pTrcHis	E. coli B834(DE3)	~42	~4.5 - 12.5
pRSET	E. coli B834(DE3)	~48	~14.8

* DCB unit is a measure of dechlorination activity toward 1,4-dichlorobutane (DCB).
‡ *E. coli* AG 1 chemically competent cells were purchased from Stratagene (La Jolla, CA, USA); *E. coli* TOP 10F' chemically competent cells were purchased from Invitrogen (Carlsbad, CA, USA); *E. coli* GI 174 cells were purchased from Invitrogen (Carlsbad, CA) and were made electro-competent according to the supplier's instructions.

Example 5

Modified *Rhodococcus* Dehalogenase

Since the *Rhodococcus* dehalogenase being produced by the TrcHis RDhl construct had been modified with additional amino acids at both the amino and carboxy termini, plasmid constructs were generated to test the effects each of these modifications might have on the activity of the enzyme. The amino terminal poly-histidine tail was eliminated by enzymatic digestion of the pTrcHis RDhl 18-3 plasmid with *Nco*I and *Age*I and the ligation of a 17 bp oligo into the resulting gap.

The DNA sequences of the oligo pairs are as follows:

RDhl Delta His-6-F
5'-CATGGGTGAAATAGGTA-3'

RDhl Delta His-6-R
5'-CCGGTACCTATTTCACC-3'

Using standard molecular biology protocols, the His-6-F and His-6-R oligonucleotides were annealed, ligated into the digested 18-3 construct, and transformed into competent E. coli TOP10 F' cells. Transformed colonies were selected by growth on LB/Amp agar plates. The resulting amino terminal sequence was:

```
-12  -11   3
ATG GGT GAA ATA GGT
Met  Gly  Ile
```

(shown with the amino acid numbering of the original, unmodified sequence).

Digestion and re-ligation resulted in a construct in which the Ala-293 Ser-294 sequence (Figure 2) became an Ala-293 Arg-294 sequence. Following the Arg-encoding codon is a stop codon which corresponds to the TGA nucleotide tri-mer at bases 927-979 in the original sequence.

The carboxy terminus EXFLAG was eliminated by digesting pTrcHis RDhl 18-3 with *Avr* II and *Nhe*I and re-ligating the plasmid.

Individual clones were screened by enzymatic digestion and gel electrophoresis. Candidate clones were grown at 37° C in 5 mL cultures, induced with IPTG, and lysed by sonication. The lysates were analyzed by PAGE, Western blot, and chloride detection assay. Those clones lacking the amino terminal poly-histidines or the carboxy terminal EXFLAG demonstrated catalytic activity equal to the original construct.

Example 6

Construction of pTrcHis RDhl-S-Tag and pRSET RDhl-S-Tag

Material and Methods:

CTERM S-Tag F (forward) and CTERM S-Tag R (reverse) are two primers that were designed to change the FLAG polypeptide – an 11 amino acid sequence – to the S-Tag polypeptide, a 15 amino acid sequence. The sequences of these oligonucleotides are as follows (each strand of the S-Tag fragment is underlined):

---Avr II---

CTERM S-Tag F 5'-CTA GGT GAC AAA GAA ACC GCT GCT GCT AAA
---Nsp V---
TTC GAA CGC CAG CAC ATG GAC AGC AAA TAA
GTT TAA ACA TCA TTCCAATTGC

---Not I---

CTERM S-Tag R 5'-GGCCGCAATTGGAATGATGTTTA AAC TTA TTT GCT
---Nsp V---
GTC CAT GTG CTG GCG TTC GAA TTT AGC AGC AGC
GGT TTC TTT GTCAC

Construction of pTrcHis RDhl-S-Tag

To generate plasmid pTrcHis RDhl-S-Tag, the plasmid pTrcHis RDhl clone 18-3 was digested with the restriction enzymes *Avr* II and *Not* I and ligated with the S-Tag fragment (the S-Tag fragment was prepared by annealing primer CTERM S-Tag F and primer CTERM S-Tag R together at room temperature). The new construct, pTrcHis RDhl-S-Tag, was incorporated into *E. coli* AG 1 competent cells (from Stratagene of La Jolla, CA, USA) and ampicillin resistant colonies were picked. Plasmids containing the S-Tag fragment were identified by analytical restriction enzyme digestion.

Construction of pRSET RDhl-S-Tag

The same procedure that was used to construct pTrcHis RDhl-S-Tag was also used to construct pRSET RDhl-S-Tag, but instead starting with the plasmid pRSET RDhl clone 16-4 – digested with the restriction enzymes, *Avr* II and *Not* I – and ligating that construct to the S-Tag fragment described above.

Semi-purified rRDhl (the EXFLAG-tagged protein derived from TrcHis RDhl Clone 18-3) was compared kinetically with semi-purified RDhl-S-Tag protein produced in this example. Chloride-releasing activity was examined at TCP concentrations ranging from 0mM to 5mM. As shown in Figure 19, the S-Tag-modified protein exhibited a consistent increase of about 15% in Vmax over the EXFLAG-modified protein. The S-Tag protein also exhibits a ~25% lower Km for TCP than does the EXFLAG protein. These results confirm that changes at the C-terminal end of the TDhl enzyme can be used to modulate and improve activity of the enzyme.

Example 7

Reactor Design and Performance

Reactor Set-Up:

A bench-scale reactor was assembled using all 316 stainless steel with ¼ inch ID, in a shell and tube design. Reactor inlet and outlet tubing were also stainless steel. A Lauda circulating water bath (with a thermostat) was used to maintain the reactor at 30°C. The reactor was packed with immobilized rRDhl enzyme running in an up-flow direction. The immobilized rRDhl enzyme was first prepared by loading a partially purified enzyme preparation (approximately 70% purity by SDS-PAGE) onto PEI-impregnated alumina (ISP 4000 grade from UOP) which had been pretreated with 25% (w/v) glutaraldehyde for 2 hours; this was followed by extensive washing with distilled water. Sufficient protein was introduced to the alumina to provide 300 mg protein (by Lowry method) per gm of support. Binding was allowed to occur overnight at room temperature. Bound enzyme activity was estimated by measuring unbound enzyme activity in the bathing solution or by final absorbance at 280 nm.

The immobilized rRDhl enzyme was transferred to the reactor using 2 mm glass beads as spacers at the inlet and outlet. Flow was initiated using an aqueous feed of a pre-warmed 10 mM sodium phosphate/10 μM EDTA buffer (pH 7.0). After several hours of wash to remove any unbound enzyme, the aqueous feed was saturated with 1,2,3-trichloropropane (TCP) and delivered as a continuously stirred solution at a flow rate of 0.15 mL/min. The reactor was allowed to equilibrate for ~ 2 residence times before sampling the inlet and outlet streams for analysis of reactant TCP and product 2,3-dichloro-1-propanol (DCH) concentrations by GC. In order to prepare the samples for GC, each sample was first saturated with sodium sulfate and then extracted with chloroform (2 volumes) containing 10mM each of two internal standards (1,1,1,2-tetrachloroethane and 3-chloro-1-propanol). TCP and DCH levels were then estimated from the GC data using the internal standard method and the productivity (the percent yield per volume per time) was calculated therefrom. This initial productivity was used as a measure of initial enzyme activity.

Productivity of Bench Scale rRDhl Bioreactor

The bioreactor was run continuously for a period of three months, with periodic sampling of the inlet and outlet streams according to the above-described method. Volumetric productivity (product weight per fluid volume per minute) of the enzyme was determined at each time point and the percent conversion fell from about 60% to about 40% over this time period. Measurements are presented in Figure 16. Based on this data, the

half life of the immobilized enzyme was estimated to be about 3500 hours. This places the immobilized dehalogenase among the most stable protein catalysts yet reported.

Example 8

Directed Evolution of Dehalogenases by EPPCR

Error-Prone PCR Mutagenesis was performed upon *Age*I- and *Nhe*I-digests of plasmid pTrc/His RDhl 18-3 which had been purified by agarose gel electrophoresis and extracted from the gel. The EPPCR products were ligated into expression vectors having *Age*I and *Nhe*I cutting sites. The resulting plasmids were transformed into competent AGI cells which were grown into colonies on ampicillin-supplemented agar. The resulting cell clone "pTric/His RDhl" EPPCR library was tested using the procedure for measuring RDhl enzyme activity by detection of pH change, as follows: wells B1 to H12 of a 96-well microplate, each containing 200µL SOB/Amp broth, was inoculated with a single colony from the pTrc/His RDhl EPPCR library; wells A1-A6 contain only media as a negative control, and wells A7-A12 were inoculated with wild type pTrc/His RDhl 18-3 colonies as a positive control. Representative results are presented in Figures 17 and 18.

The results demonstrate that most of the clones produced by EPPCR mutagenesis exhibit activities equal to or less than the activity range for the wild-type RDhl produced by TrcHis RDhl Clone 18-3. However, in each case in which 84 random clones from an EPPCR library were analyzed for dehalogenase activity, a few on each 96-well plate exhibited activity significantly higher than that of the wild-type enzymes.

On February 3, 1998, the three plasmids, pTrcHis RDhl clone 18-3, pRSET RDhl clone 16-4, and pTrxFus RDhl clone 4, were deposited with the American Type Culture Collection (ATCC) in accordance with the Budapest Treaty and were respectively given the following designations: ATCC 209609, ATCC 209610, and ATCC 209611. On February 3, 1998, the cell culture *E. coli* TrxFus RDhl clone 4, was deposited with the American Type Culture Collection (ATCC) in accordance with the Budapest Treaty and was given the following designation: ATCC 202087.

On January 30, 1998, the two cell cultures, *E. coli* TrcHis RDhl clone 18-3 and *E. coli* RSET RDhl clone 16-4 were deposited with the American Type Culture Collection (ATCC) in accordance with the Budapest Treaty and were respectively given the following designations: ATCC 202086 and ATCC 202085.

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of this specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

GENERATION INFORMATION:

APPLICANT:

NAME: The Dow Chemical Company
STREET: 1790 Bldg. Washington Street
CITY: Midland
STATE: MI
COUNTRY: U.S.A.
POSTAL CODE: 48674
TELEPHONE: 517-636-1687
TELEFAX: 517-638-9786

TITLE OF INVENTION: Recombinant Haloaliphatic Dehalogenases

NUMBER OF SEQUENCES: 26

COMPUTER READABLE FROM:

MEDIUM TYPE: 3-1/2" Floppy disk
COMPUTER: IBM PC compatible
OPERATING SYSTEM: MS-DOS
SOFTWARE: PatentIn

INFORMATION FOR SEQ ID NO:1:

SEQUENCE CHARACTERISTICS

LENGTH: 305
TYPE: amino acid
STRANDEDNESS: single
TOPOLOGY: linear

ORIGINAL SOURCE:

ORGANISM: Rhodococcus rhodocrous
INDIVIDUAL ISOLATE: TDTM003

FEATURE:

NAME/KEY: RDhl Enzyme
LOCATION: 1..292

FEATURE:

NAME/KEY: Carboxy-terminal EXFLAG tail
LOCATION: 295..305

FEATURE:

NAME/KEY: Amino-terminal poly-His tail
LOCATION: -10..-1

SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Gly Gly Ser His His His His His His Gly Met Ser Glu Ile Gly
-12     -10                 -5                  -1   1

Thr Gly Phe Pro Phe Asp Pro His Tyr Val Glu Val Leu Gly Glu Arg
 5                  10

Met His Tyr Val Asp Val Gly Pro Arg Asp Gly Thr Pro Val Leu Phe
```

Leu His Gly Asn Pro Thr Ser Ser Tyr Leu Trp Arg Asn Ile Ile Pro

His Val Ala Pro Ser His Arg Trp Ile Ala Pro Asp Leu Ile Gly Met

Gly Lys Ser Asp Lys Pro Asp Leu Asp Tyr Phe Phe Asp Asp His Val

Arg Tyr Leu Asp Ala Phe Ile Glu Ala Leu Gly Leu Glu Glu Val Val

Leu Val Ile His Asp Trp Gly Ser Ala Leu Gly Phe His Trp Ala Lys

Arg Asn Pro Glu Arg Val Lys Gly Ile Ala Cys Met Glu Phe Ile Arg

Pro Ile Pro Thr Trp Asp Glu Trp Pro Glu Phe Ala Arg Glu Thr Phe

Gln Ala Phe Arg Thr Ala Asp Val Gly Arg Glu Leu Ile Ile Asp Gln

Asn Ala Phe Ile Glu Gly Val Leu Pro Lys Cys Val Val Arg Arg Leu

Thr Glu Val Glu Met Asp His Tyr Arg Glu Pro Phe Leu Lys Pro Val

Asp Arg Glu Pro Leu Trp Arg Phe Pro Asn Glu Ile Pro Ile Ala Gly

Glu Pro Ala Asn Ile Val Ala Leu Val Glu Ala Tyr Met Asn Trp Leu

His Gln Ser Pro Val Pro Lys Leu Leu Phe Trp Gly Thr Pro Gly Val

Leu Ile Pro Pro Ala Glu Ala Ala Arg Leu Ala Glu Ser Leu Pro Asn

Cys Lys Thr Val Asp Ile Gly Pro Gly Leu His Tyr Leu Gln Glu Asp

```
Asn Pro Asp Leu Ile Gly Ser Glu Ile Ala Arg Trp Leu Pro Gly Leu
                                                290

Ala Ser Lys Leu Gly Asp Tyr Lys Asp Asp Asp Asp Lys
        295                 300                 305
```

SEQ ID NO:2
RDhl Fig.2 DNA

CC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG TCT GAA ATA 47
GGT ACC GGT TTT CCC TTC GAC CCT CAT TAT GTG GAA GTC CTG GGC GAG
CGT ATG CAC TAC GTC GAT GTT GGA CCG CGG GAT GGC ACG CCT GTG CTG
TTC CTG CAC GGT AAC CCG ACC TCG TCC TAC CTG TGG CGC AAC ATC ATC
CCG CAT GTA GCA CCG AGT CAT CGG TGC ATT GCT CCA GAC CTG ATC GGG
ATG GGA AAA TCG GAC AAA CCA GAC CTC GAT TAT TTC TTC GAC GAC CAC
GTC CGC TAC CTC GAT GCC TTC ATC GAA GCC TTG GGT TTG GAA GAG GTC
GTC CTG GTC ATC CAC GAC TGG GGC TCA GCT CTC GGA TTC CAC TGG GCC
AAG CGC AAT CCG GAA CGG GTC AAA GGT ATT GCA TGT ATG GAA TTC ATC
CGG CCT ATC CCG ACG TGG GAC GAA TGG CCG GAA TTC GCC CGT GAG ACC
TTC CAG GCC TTC CGG ACC GCC GAC GTC GGC CGA GAG TTG ATC ATC GAT
CAG AAC GCT TTC ATC GAG GGT GTG CTC CCG AAA TGC GTC GTC CGT CCG
CTT ACG GAG GTC GAG ATG GAC CAC TAT CGC GAG CCC TTC CTC AAG CCT
GTT GAC CGA GAG CCA CTG TGG CGA TTC CCC AAC GAG ATC CCC ATC GCC
GGT GAG CCC GCG AAC ATC GTC GCG CTC GTC GAG GCA TAC ATG AAC TGG
CTG CAC CAG TCA CCT GTC CCG AAG TTG TTG TTC TGG GGC ACA CCC GGC
GTA CTG ATC CCC CCG GCC GAA GCC GCG AGA CTT GCC GAA AGC CTC CCC
AAC TGC AAG ACA GTG GAC ATC GGC CCG GGA TTG CAC TAC CTC CAG GAA
GAC AAC CCG GAC CTT ATC GGC AGT GAG ATC GCG CGC TGG CTC CCC GGA
CTC GCT AGC GGC CTA GGT GAC TAC AAG GAC GAT GAT GAC AAA TAA TGA
GCGGCCGC AAGCTT

SEQ ID NO:3
TCCH aa

Met Ser Leu Gly Ala Lys Pro Phe Gly Glu Lys Lys Phe Ile Glu Ile
Lys Gly Arg Arg Met Ala Tyr Ile Asp Glu Gly Thr Gly Asp Pro Ile
Leu Phe Gln His Gly Asn Pro Thr Ser Ser Tyr Leu Trp Arg Asn Ile
Met Pro His Cys Ala Gly Leu Gly Arg Leu Ile Ala Cys Asp Leu Ile
Gly Met Gly Asp Ser Asp Lys Leu Asp Pro Ser Gly Pro Glu Arg Tyr
Ala Tyr Ala Glu His Arg Asp Tyr Leu Asp Ala Leu Trp Glu Ala Leu
Asp Leu Gly Asp Arg Val Val Leu Val Val His Asp Trp Gly Ser Ala
Leu Gly Phe Asp Trp Ala Arg Arg His Arg Glu Arg Val Gln Gly Ile
Ala Tyr Met Glu Ala Ile Ala Met Pro Ile Glu Trp Ala Asp Phe Pro
Glu Gln Asp Arg Asp Leu Phe Gln Ala Phe Arg Ser Gln Ala Gly Glu
Glu Leu Val Leu Gln Asp Asn Val Phe Val Glu Gln Val Leu Pro Gly
Leu Ile Leu Arg Pro Leu Ser Glu Ala Glu Met Ala Ala Tyr Arg Glu
Pro Phe Leu Ala Ala Glu Ala Arg Arg Pro Thr Leu Ser Trp Pro Arg
Gln Ile Pro Ile Ala Gly Thr Pro Ala Asp Val Val Ala Ile Ala Arg
Asp Tyr Ala Gly Trp Leu Ser Glu Ser Pro Ile Pro Lys Leu Phe Ile
Asn Ala Glu Pro Gly Ala Leu Thr Thr Gly Arg Met Arg Asp Phe Cys
Arg Thr Trp Pro Asn Gln Thr Glu Ile Thr Val Ala Gly Ala His Phe
Ile Gln Glu Asp Ser Pro Asp Glu Ile Gly Ala Ala Ile Ala Ala Phe
Val Arg Arg Leu Arg Pro Ala

SEQ ID NO:4
Rlucif aa.

Met Thr Ser Lys Val Tyr Asp Pro Glu Gln Arg Lys Arg Met Ile Thr
Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser
Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile
Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val

Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly
Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp
His Tyr Lys Tyr Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys
Lys Ile Ile Phe Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His
Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu
Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu
Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu
Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg
Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu
Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro
Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr
Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu
Ser Asp Pro Gly Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys
Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln
Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu
Arg Val Leu Lys Asn Glu Gln

SEQ ID NO:5
XDhl aa.

Met Ile Asn Ala Ile Arg Thr Pro Asp Gln Arg Phe Ser Asn Leu Asp
Gln Tyr Pro Phe Ser Pro Asn Tyr Leu Asp Asp Leu Pro Gly Tyr Pro
Gly Leu Arg Ala His Tyr Leu Asp Glu Gly Asn Ser Asp Ala Glu Asp
Val Phe Leu Cys Leu His Gly Glu Pro Thr Trp Ser Tyr Leu Tyr Arg
Lys Met Ile Pro Val Phe Ala Glu Ser Gly Ala Arg Val Ile Ala Pro
Asp Phe Phe Gly Phe Gly Lys Ser Asp Lys Pro Val Asp Glu Glu Asp
Tyr Thr Phe Glu Phe His Arg Asn Phe Leu Leu Ala Leu Ile Glu Arg
Leu Asp Leu Arg Asn Ile Thr Leu Val Val Gln Asp Trp Gly Gly Phe
Leu Gly Leu Thr Leu Pro Met Ala Asp Pro Ser Arg Phe Lys Arg Leu
Ile Ile Met Asn Ala Cys Leu Met Thr Asp Pro Val Thr Gln Pro Ala
Phe Ser Ala Phe Val Thr Gln Pro Ala Asp Gly Phe Thr Ala Trp Lys
Tyr Asp Leu Val Thr Pro Ser Asp Leu Arg Leu Asp Gln Phe Met Lys
Arg Trp Ala Pro Thr Leu Thr Glu Ala Glu Ala Ser Ala Tyr Ala Ala
Pro Phe Pro Asp Thr Ser Tyr Gln Ala Gly Val Arg Lys Phe Pro Lys
Met Val Ala Gln Arg Asp Gln Ala Cys Ile Asp Ile Ser Thr Glu Ala
Ile Ser Phe Trp Gln Asn Asp Trp Asn Gly Gln Thr Phe Met Ala Ile
Gly Met Lys Asp Lys Leu Leu Gly Pro Asp Val Met Tyr Pro Met Lys
Ala Leu Ile Asn Gly Cys Pro Glu Pro Leu Glu Ile Ala Asp Ala Gly
His Phe Val Gln Glu Phe Gly Glu Gln Val Ala Arg Glu Ala Leu Lys
His Phe Ala Glu Thr Glu

SEQ ID NO:6
RDhl 5.4

GGTTCCATGG GNTTYCCNTT YGAYCCNCAY TA

SEQ ID NO:7
RDhl 3.12

CAGAGCTAGC GAGTCCGGGG AGCCAGCG

SEQ ID NO:8
RDhl 5.7

CGTACATATG GCCATGGGGG GTTCTCATCA TCATCATCAT CATGGTATGT CTGAAATAGG
TACCGGTTTT CCCTTCGACC CTCATTA

SEQ ID NO:9
RDhl 3.13

GATGACAAAT AATGAGCGGC CGCAAGCTTG TAC

SEQ ID NO:10
Trx2++

CCGGGGATCC CATGGCTTCT GAAATACGTA CCGGTTTTCC CTTCGACCCT CATTA

SEQ ID NO:11
Trx-

TCGACTGCAG GCGGCCGCTC ATTATTTGTC ATC

SEQ ID NO:12
Dhl Seq 7

CCTGTCCCGA AGTTGTTG

SEQ ID NO:13
Dhl Seq 8

CGGGCCGATC TCCACTG

SEQ ID NO:14
Dhl Seq 11

TGCTCCAGAC CTGATCG

SEQ ID NO:15
Dhl Seq 12

TCTGATCGAT GATCAAC

SEQ ID NO:16
Dhl Seq 13

TCCCGACGTG GACGAATG

SEQ ID NO:17
Dhl Seq 14

GAGCGCGACG ATGTTCGC

SEQ ID NO:18
Dhl Seq 15

CACCCGGCGT ACTGATCC

SEQ ID NO:19
Dhl Seq 18

GAGACCGGTC AGCATTCC

SEQ ID NO:20
PROK-Seq1

GAGCGGATAA CAATTTCA

SEQ ID NO:21
PROK-Seq2

TCTCATCCGC CAAAACAG

SEQ ID NO:22
EXFLAG linker

GAATTCAGCC ATGGCATAAG CTTTCTAGAC TCGAGGGAGC TAGCGGCCTA GGTGACTACAA
GGACGATGAT GACAAATAAT GAGCGGCCGC TAGCTT

SEQ ID NO:23
RDhl Delta His-6-F

CATGGGTGAA ATAGGTA

SEQ ID NO:24
RDhl Delta His-6-R

CCGGTACCTA TTTCACC

SEQ ID NO:25
CTERM S-Tag F

CTAGGTGACAAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCAAATAAGTTTAAACATCA
TTCCAATTGC

SEQ ID NO:26
CTERM S-Tag R

GGCCGCAATTGGAATGATGTTTAAACTTATTTGCTGTCCATGTGCTGGCGTTCGAATTTAGCAGCAGCGGTT
TCTTTGTCAC

What is Claimed is:

1. An enzyme capable of converting a halogenated aliphatic hydrocarbon to a halohydrin, said enzyme contains a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2.

2. The enzyme of Claim 1 wherein said enzyme contains an amino acid sequence which is at least about 90% to 100% homologous with the amino acid sequence of residues 1-292 of Figure 2.

3. The enzyme of Claim 2 wherein said enzyme contains an amino acid sequence which is at least about 95% homologous with the amino acid sequence of residues 1-292 of Figure 2.

4. A DNA sequence from which can be expressed a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2, said polypeptide being capable of converting a halogenated aliphatic hydrocarbon to a halohydrin.

5. The DNA sequence of Claim 4 wherein said polypeptide is at least about 90% to 100% homologous with the amino acid sequence of residues 1-292 of Figure 2.

6. The DNA sequence of Claim 5 wherein said polypeptide is at least about 95% homologous with the amino acid sequence of residues 1-292 of Figure 2.

7. A DNA sequence containing a polynucleotide substantially homologous with the nucleotide sequence of bases 37-912 of Figure 2, said polynucleotide being capable of expressing a polypeptide which is able to convert a halogenated aliphatic hydrocarbon to a halohydrin.

8. The DNA sequence of Claim 7 wherein said polynucleotide is at least 90% to 100% homologous with the nucleotide sequence of bases 37-912 of Figure 2.

9. The DNA sequence of Claim 8 wherein said polynucleotide is at least 95% homologous with the nucleotide sequence of bases 37-912 of Figure 2.

10. A microorganism containing a recombinant plasmid wherein the plasmid is capable of directing the synthesis of an enzyme containing a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2.

11. The microorganism of Claim 10 wherein the microorganism is of the genus *Escherichia, Pichia, Bacillus, Saccharomyces, Pseudomonas, Rhodococcus, Actinomyces, or Aspergillus.*

12. The microorganism of Claim 11 wherein the microorganism is of the genus *Escherichia*.

13. An expression construct containing a DNA sequence which encodes a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2.

14. An immobilized enzyme having a haloalkane dehalogenase enzyme which has haloaliphatic dehalogenase activity and is attached to a solid support.

15. The enzyme of Claim 14 wherein the enzyme is capable of hydrolytically removing at least one halogen substituent from a molecule or group selected from the group consisting of halogenated aliphatic hydrocarbon, halogenated aliphatic alcohol, and halogenated aliphatic polyol molecules and groups.

16. The enzyme of Claim 15 wherein said molecule or group has at least one halogen atom and 2 to 10 carbon atoms, each of said carbon atoms being independently substituted with one or fewer of said halogen atoms, provided that when said molecule or group is an alcohol or polyol, no carbon atom thereof having a hydroxy substituent also has a halogen substituent.

17. The enzyme of Claim 16 wherein said molecule or group contains at least 2 halogen atoms.

18. The enzyme of Claim 17 wherein said molecule or group is a 1,2-dihalo molecule or group.

19. The enzyme of Claim 17 wherein said molecule or group is selected from the group consisting of the 1,2-dihaloethane, 1,2-dihalopropane, 1,2-dihalobutane, and 1,2,3-trihalopropane.

20. The enzyme of Claim 19 wherein said molecule or group is respectively selected from the group consisting of 1,2-dichloroethane, 1,2-dichloropropane, 1,2-dichlorobutane, 1,2-dibromo-3-chloropropane, and 1,2,3-trichloropropane.

21. The enzyme of Claim 20 wherein said molecule or group is converted to at least one product molecule or product group which is selected from 2-chloro-ethanol, 1-chloro-2-propanol, 2-chloro-1-propanol, 1-chloro-2-butanol, 2-chloro-1-butanol, 1-bromo-3-chloro-2-propanol, 2-bromo-3-chloro-1-propanol, 2,3-dibromo-1-propanol, 1,2-dichloro-3-propanol, and 1,3-dichloro-2-propanol.

22. The enzyme of Claim 14 wherein said haloalkane dehalogenase is obtained from a *Rhodococcus*.

23. A process for preparing an enzyme containing a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2, which comprises the steps of:

1) providing a DNA segment comprising a polynucleotide capable of expressing said polypeptide,

2) inserting said DNA segment into an expression construct,

3) transfecting a host cell with said expression construct, and

4) providing the host cell with an environment in which it expresses said polypeptide.

24. The process of Claim 23 further comprising a step of purifying said enzyme after step 4 of said process.

25. A process for preparing an immobilized enzyme containing a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2, covalently linked to a solid support, comprising the steps of:

1) providing an enzyme containing a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2,

2) providing a solid support which is attached to linker having at least one reactive group, and

3) contacting said enzyme with said linker under biocompatible conditions in which said reactive group reacts with an amino, carboxy, hydroxy, or sulfhydryl group covalently attached to said polypeptide to form a covalent attachment.

26. The process of Claim 25 wherein said linker has at least one group selected from among the dialdehydes, diacids, diamines, diisocyanates, cyanates, diimides, and carbodiimides, provided that a diamine is not used in conjunction with a carbodiimide.

27. The immobilized enzyme produced according to the process of Claim 25.

28. A process of converting a halogenated aliphatic hydrocarbon to an alcohol or a halohydrin comprising the steps of:

1) providing an enzyme containing a polypeptide substantially homologous with the amino acid sequence of residues 1-292 of Figure 2,

2) providing a solid support which is attached to a linker having at least one reactive group,

3) contacting said enzyme with said linker under biocompatible conditions in which said reactive group reacts with an amino, carboxy, hydroxy, or sulfhydryl group covalently attached to said polypeptide to create a covalent attachment and form an immobilized enzyme, and

4) contacting said immobilized enzyme with a halogenated aliphatic hydrocarbon under conditions in which said enzyme can convert the halogenated aliphatic hydrocarbon to an alcohol or halohydrin.

29. The process of Claim 28 wherein said enzyme is the enzyme of Claim 2 or 3.

30. An enzyme according to any one of Claims 1-3 wherein said enzyme is a fusion protein having one or two, terminal polypeptide tails.

31. The enzyme of Claim 30 wherein said fusion protein has a single amino-terminal tail of up to 30 amino acids which contains a sequence of at least six contiguous histidine residues.

32. The enzyme of Claim 30 wherein said fusion protein has a single carboxy-terminal tail of up to 150 amino acids.

33. The enzyme of Claim 32 wherein said carboxy-terminal tail is hydrophilic.

34. The enzyme of Claim 30 wherein said fusion protein has both an amino-terminal tail of up to 30 amino acids and a carboxy-terminal tail of up to 150 amino acids.

35. The enzyme of Claim 34 wherein said amino terminal tail contains a sequence of at least six contiguous histidine residues.

36. The enzyme of Claim 34 wherein said carboxy-terminal tail contains a polypeptide selected from the group consisting of the EXFLAG polypeptide, the S-Tag polypeptide, hexahistidine-sequence-containing polypeptides, and cellulose binding domains.

37. An enzyme having halogenated aliphatic dehalogenase activity whose DNA has been derived, by a directed evolution process, from a related DNA sequence, wherein said enzyme has a dehalogenating activity which is greater than that of the related DNA sequence.

38. The enzyme according to Claim 37 wherein said related DNA sequence encodes a wild-type or recombinant dehalogenase.

39. The enzyme according to Claim 38 wherein said related DNA sequence encodes a wild-type or recombinant haloalkane dehalogenase.

40. The enzyme according to Claim 39 wherein said related DNA sequence encodes a wild-type or recombinant *Rhodococcus* haloalkane dehalogenase.

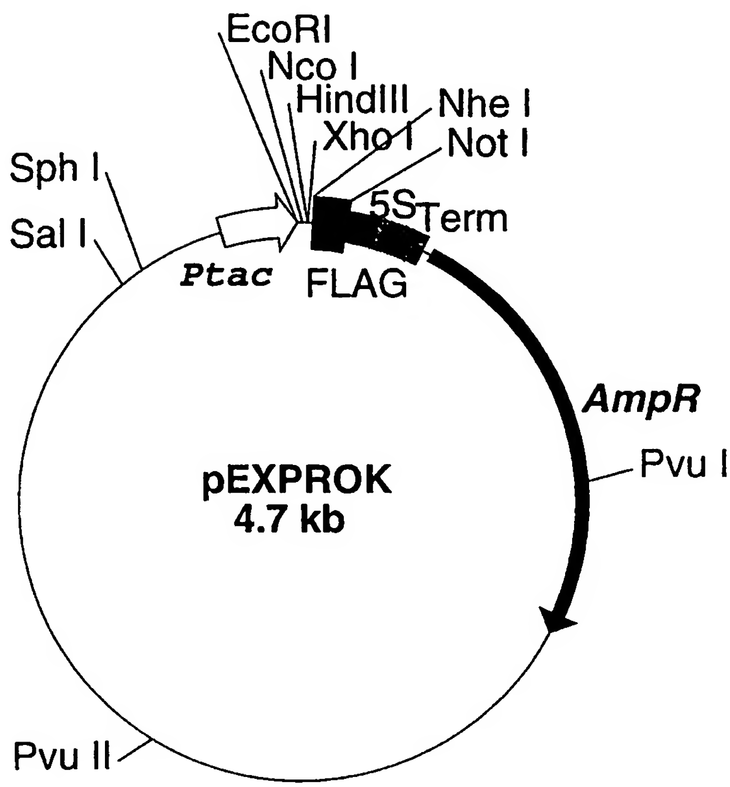
41. The enzyme according to Claim 37 wherein said directed evolution process involves performing error-prone PCR.

42. The enzyme according to Claim 37 wherein the enzyme is a fusion protein having one or two terminal polypeptide tails.

43. The enzyme according to Claim 42 wherein one tail or both tails have been modified by a directed evolution process.

44. An expression vector selected from the group consisting of ATCC 209609, ATCC 209610, and ATCC 209611.

45. A cell culture selected from the group consisting of ATCC 202085, ATCC 202086, and ATCC 202087.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Not I
Sph I
Sal I
5S
Term
Ptac
FLAG
AmpR
Pvu I
pEXPROK
4.7 kb
Pvu II

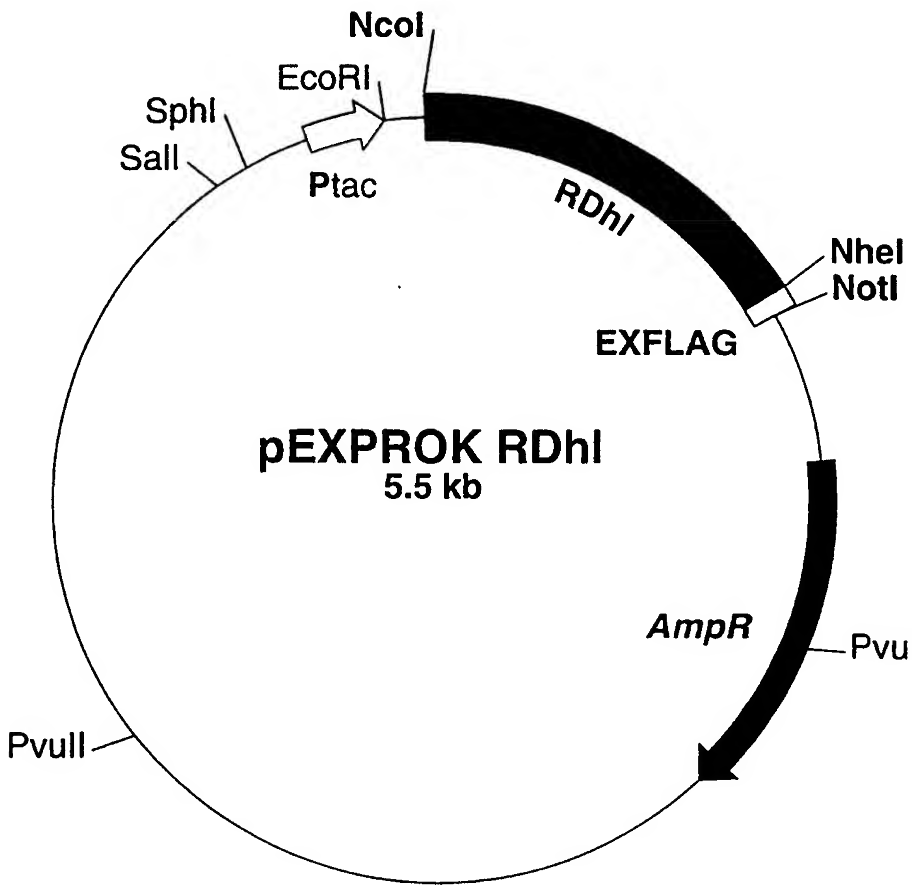
Fig. 1

Fig. 2A

```
1                                   30                                            60
Nco I                                *                    *           *           *
ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG TCT GAA ATA GGT ACC GGT TTT CCC TTC GAC CCT
 M   G   G   S   H   H   H   H   H   H   G   M   S   E   I   G   T   G   F   P   F   D   P
        -10  modified poly-His sequence              1                                10

                            90                               120
 *           *               *           *           *         *                 *
CAT TAT GTG GAA GTC CTG GGC GAG CGT ATG CAC TAC GTC GAT GTT GGA CCG CGG GAT GGC ACG CCT GTG
 H   Y   V   E   V   L   G   E   R   M   H   Y   V   D   V   G   P   R   D   G   T   P   V
                                 20                                      30

             150                                     180
 *            *              *           *            *              *           *
CTG TTC CTG CAC GGT AAC CCG ACC TCG TCC TAC CTG TGG CGC AAC ATC ATC CCG CAT GTA GCA CCG AGT
 L   F   L   H   G   N   P   T   S   S   Y   L   W   R   N   I   I   P   H   V   A   P   S
                         40                                  50

210                                      240                                      270
 *           *           *                *              *           *             *
CAT CGG TGC ATT GCT CCA GAC CTG ATC GGG ATG GGA AAA TCG GAC AAA CCA GAC CTC GAT TAT TTC TTC
 H   R   W   I   A   P   D   L   I   G   M   G   K   S   D   K   P   D   L   D   Y   F   F
         60                                      70                                      80

                            300                                330
     *           *           *           *           *           *                 *
GAC GAC CAC GTC CGC TAC CTC GAT GCC TTC ATC GAA GCC TTG GGT TTG GAA GAG GTC GTC CTG GTC ATC
 D   D   H   V   R   Y   L   D   A   F   I   E   A   L   G   L   E   E   V   V   L   V   I
                                     90                                  100

                 360                                 390
     *            *           *           *           *              *           *
CAC GAC TGG GGC TCA GCT CTC GGA TTC CAC TGG GCC AAG CGC AAT CCG GAA CGG GTC AAA GGT ATT GCA
 H   D   W   G   S   A   L   G   F   H   W   A   K   R   N   P   E   R   V   K   G   I   A
                         110                                 120

    420                                  450                                      480
     *               *           *        *              *           *             *
TGT ATG GAA TTC ATC CGG CCT ATC CCG ACG TGG GAC GAA TGG CCG GAA TTC GCC CGT GAG ACC TTC CAG
 C   M   E   F   I   R   P   I   P   T   W   D   E   W   P   E   F   A   R   E   T   F   Q
             130                                 140
```

Fig. 2B

```
                                             510                                                              540
        *                 *                   *                 *               *                     *                   *
GCC TTC CGG ACC GCC GAC GTC GGC CGA GAG TTG ATC ATC GAT CAG AAC GCT TTC ATC GAG GGT GTG CTC
 A   F   R   T   A   D   V   G   R   E   L   I   I   D   Q   N   A   F   I   E   G   V   L
150                                          160                                         170

                     570                                                  600
        *             *               *                 *                   *               *                 *
CCG AAA TGC GTC GTC CGT CCG CTT ACG GAG GTC GAG ATG GAC CAC TAT CGC GAG CCC TTC CTC AAG CCT
 P   K   C   V   V   R   P   L   T   E   V   E   M   D   H   Y   R   E   P   F   L   K   P
                             180                                         190

        630                                      660                                             690
         *               *             *               *                 *                 *       *
GTT GAC CGA GAG CCA CTG TGG CGA TTC CCC AAC GAG ATC CCC ATC GCC GGT GAG CCC GCG AAC ATC GTC
 V   D   R   E   P   L   W   R   F   P   N   E   I   P   I   A   G   E   P   A   N   I   V
                200                                       210

                                 720                                             750
            *           *         *               *               *               *
GCG CTC GTC GAG GCA TAC ATG AAC TGG CTG CAC CAG TCA CCT GTC CCG AAG TTG TTG TTC TGG GGC ACA
 A   L   V   E   A   Y   M   N   W   L   H   Q   S   P   V   P   K   L   L   F   W   G   T
    220                                      230                                     240

                         780                                              810
 *           *             *             *                 *                   *               *
CCC GGC GTA CTG ATC CCC CCG GCC GAA GCC GCG AGA CTT GCC GAA AGC CTC CCC AAC TGC AAG ACA GTG
 P   G   V   L   I   P   P   A   E   A   A   R   L   A   E   S   L   P   N   C   K   T   V
                                 250                                     260

            840                                          870
 *           *           *               *               *           *               *
GAC ATC GGC CCG GGA TTG CAC TAC CTC CAG GAA GAC AAC CCG GAC CTT ATC GGC AGT GAG ATC GCG CGC
 D   I   G   P   G   L   H   Y   L   Q   E   D   N   P   D   L   I   G   S   E   I   A   R
                    270                                      280

900                                 930                                                   970
 *               *           *           *               *           *                       *
TGG CTC CCC GGA CTC GCT AGC GGC CTA GGT GAC TAC AAG GAC GAT GAT GAC AAA TAA TGA GCGGCCGC AAGCTT
 W   L   P   G   L   A   S   G   L   G   D   Y   K   D   D   D   D   K   *   *
                292         |------------------------------------------->         Not I    HindIII
                    Nhe I      Avr II  EXFLAG peptide = aa 295-305
```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SalI
Ptac
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NheI
NotI
EXFLAG
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AmpR
PvuI
PvuII

Fig. 3

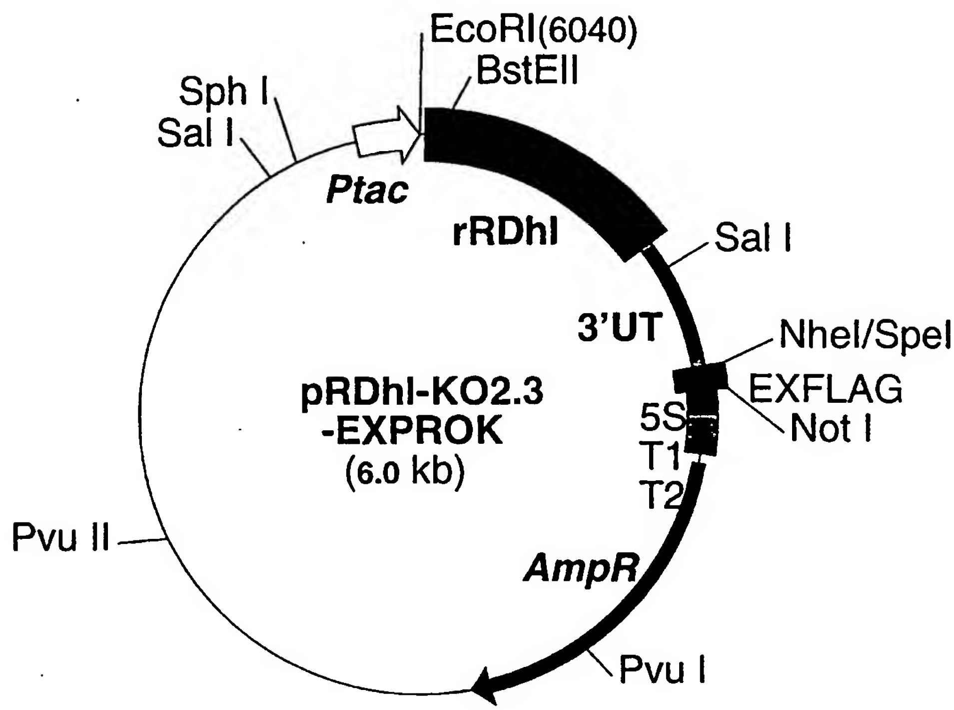
Fig. 4A

```
RDhl                          10         20        30         40         50
                               *          *         *          *          *
RDhl               SeigtgfP FdPhYvevL- G--e-RmHYv DvGprDgtpV -LfLHGnPTs SYLwRniIPh
TCCH               msLgak-P Fgekkfieik -g--rRmaYi DEGtgD-pil --fqHGnPTs SYLwRnimPh
Rlucif  MtskvydPeQ Rkr-mitgpq wwarc-kqmn vldsfinyYd sEkhaenavi F--LHGnaas SYLwRhvvPh
XDhl    MINAIRTPDQ RFSNL-DQYP FSPNYLDDLP GYPGLRAHYL DEGNSDAEDV FLCLHGEPTW SYLYRKMIPV
                 *          *         *          *          *          *
Xdhl            10         20        30         40         50         60

Rdhl          60        70           80         90        100        110
               *         *            *          *          *          *
Rdhl    vA-pshRwIA PDliGmGKSD K--P-D-lDY fFddHvryLd AfIEaLgL-e evvLVihDWG saLGfhwakr
TCCH    cAgl-gRlIA cDliGmGdSD KldPsgpErY ayaeHRdyLd ALwEaLDlgd rvvLVVhDWG saLGfdwarr
Rlucif  i-EpvARcIi PDliGmGKSg K--s-gngsY rlldHykyLt AwfEllnLpk kIifVghDWG acLafhysye
XDhl    FAESGARVIA PDFFGFGKSD K--PVDEEDY TFEFHRNFLL ALIERLDL-R NITLVVQDWG GFLGLTLPMA
        *          *          *           *          *          *  ↑       *
XDhl    70         80         90         100        110         120       130

RDhl      120        130        140        150        160        170        180
           *          *          *          *          *          *          *
RDhl    nPeRvKgiac Me-firpiP- TwdewpeFar etfqaFrtad vgreLiidqn afiegvlpkc vvrrLTEvEm
TCCH    hreRvqgiay MeAia--mPi ewadFpeqdr dlfqaFrs-q ageeLVlqdn vfveQvlpgl ilrpLsEAEm
RLucif  hqdkiK-aIv haesvvdvie swdewpdiee diAli--kse egekmVlenn ffvetmlpsk imrkLepeEf
XDhl    DPSRFKRLII MNACLMTDPV TQPAFSAFVT QPADGFTAWK Y--DLVTPSD LRLDQFMKR- WAPTLTEAEA
           *          *          *          *          *          *          *
XDhl      140        150        160        170         180        190        200
```

Fig. 4B

```
RDhl         190        200        210        220        230        240        250
               *          *          *          *          *          *          *
RDhl     dhYrePFlkp vdreplwrFP neipiagepa nivalvEAym nWlhqspvpk llfwGtpgvL ipPaeaarla
TCCH     aAYrePFlaa earrptlswP rqipiagtpa -Dvvai-Ard --yagWlses pipklfinae pGalttgrMr
RLucif   aAYlePFkek g-e--VRr-P tlswpReipl vkggkpd-vv qivrnyNayl rasddlpkmf iesDpgffsn
XDhl     SAYAAPFPDT SYQAGVRKFP KMVAQRDQAC IDIST-EAIS FWQNDWNGQT FMAIGMKDKL LGPDVMYPMK
                *          *          *           *          *          *          *
XDhl           210        220        230         240        250        260        270

RDhl         260          270        280        290
               *            *          *          *
RDhl     eslpnC-ktv dI-gpGlHyl QEdnpdligs eiarwlpgl
TCCH     dfcrtwPnqt EItvAGaHFi QEdspdeiga Aiaafvrrlr pa
RLucif   AiveG-akkf pntefvkvkg lhFsqedApd emgkyiksfv ervlkneq
XDhl     ALINGCPEPL EIADAG-HFV QEFGEQVARE ALKHFAETE
                *           *          *          *
XDhl           280         290        300        310
```

Figure 4. Alignment of the putative *Rhodococcus rhodochrous* dehalogenase (RDhl) and *Xanthobacter autotrophicus* GJ10 dehalogenase (XDhl) protein sequences with one another and with the two other most closely related members of the a/ß hydrolase enzyme family (luciferin monooxygenase from *Renilla reniformis* (RLucif) and tetrachloro-cylcohexadiene hydrolase (TCCH) from *Pseudomonas sp.*). Residues sharing identity with the XDhl sequence are capitalized. The catalytic aspartic acid residue (RDhl residue #105) is indicated by an arrow. Numbering on the overline corresponds to amino acid residue numbers of the RDhl sequence. Numbering in the underline (italicized) corresponds to the amino acid residues of the XDhl protein sequence. Initial aligment to XDhl was performed using the MacVector v.4.5.3 sequence analysis package using the pam250 matrix (Kodak Imaging Systems). Manual refinement was used to optimize overall alignment between members of the sequence group.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T2
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-EXPROK
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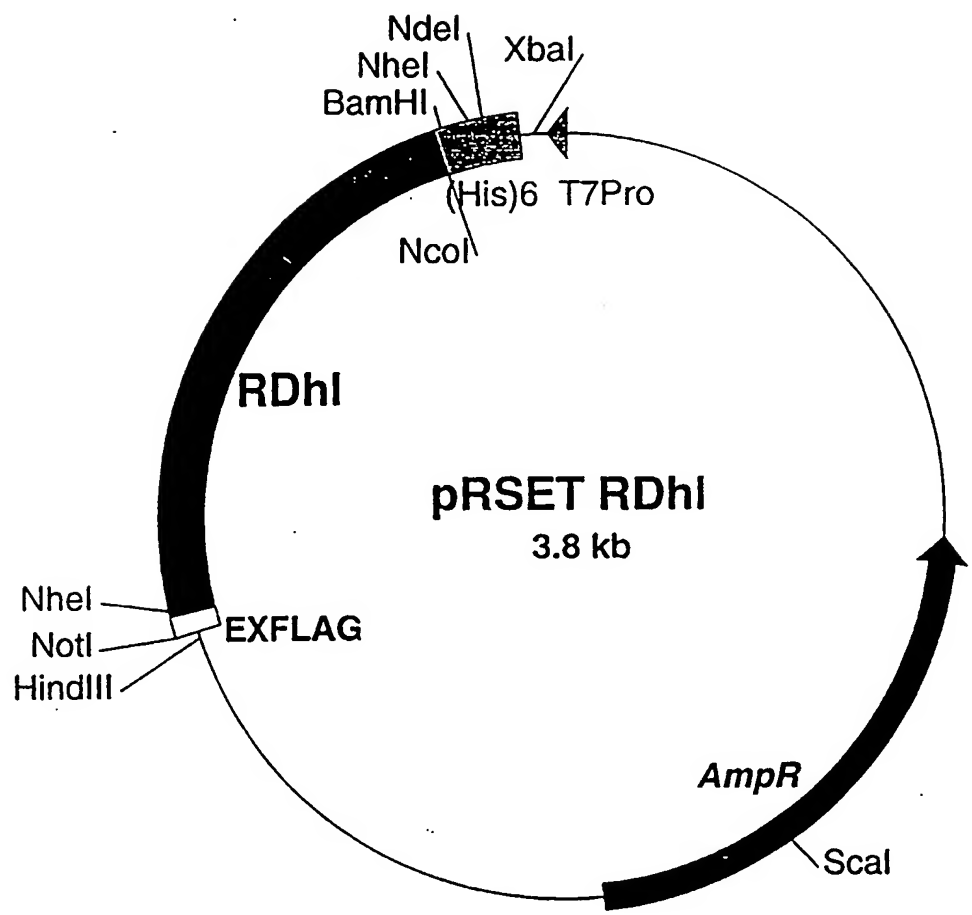
Pvu II
AmpR
Pvu I

Fig. 5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HindIII
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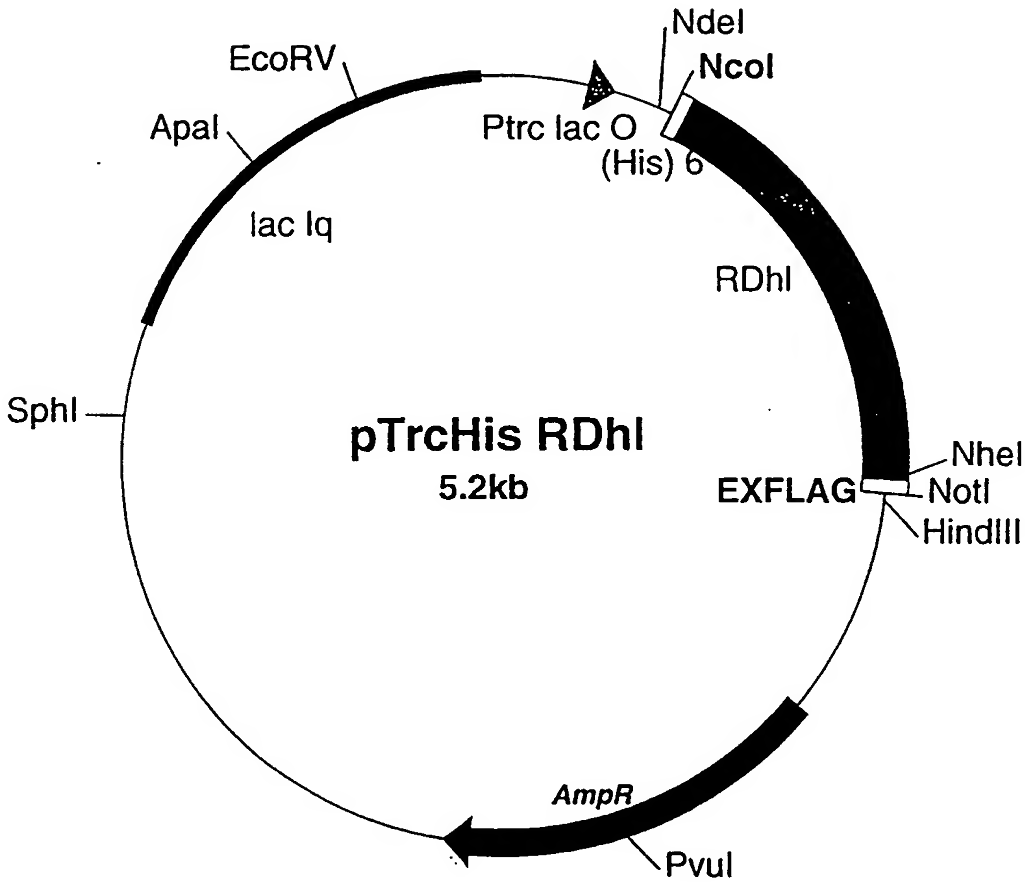
ScaI

Fig. 6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HindIII
AmpR
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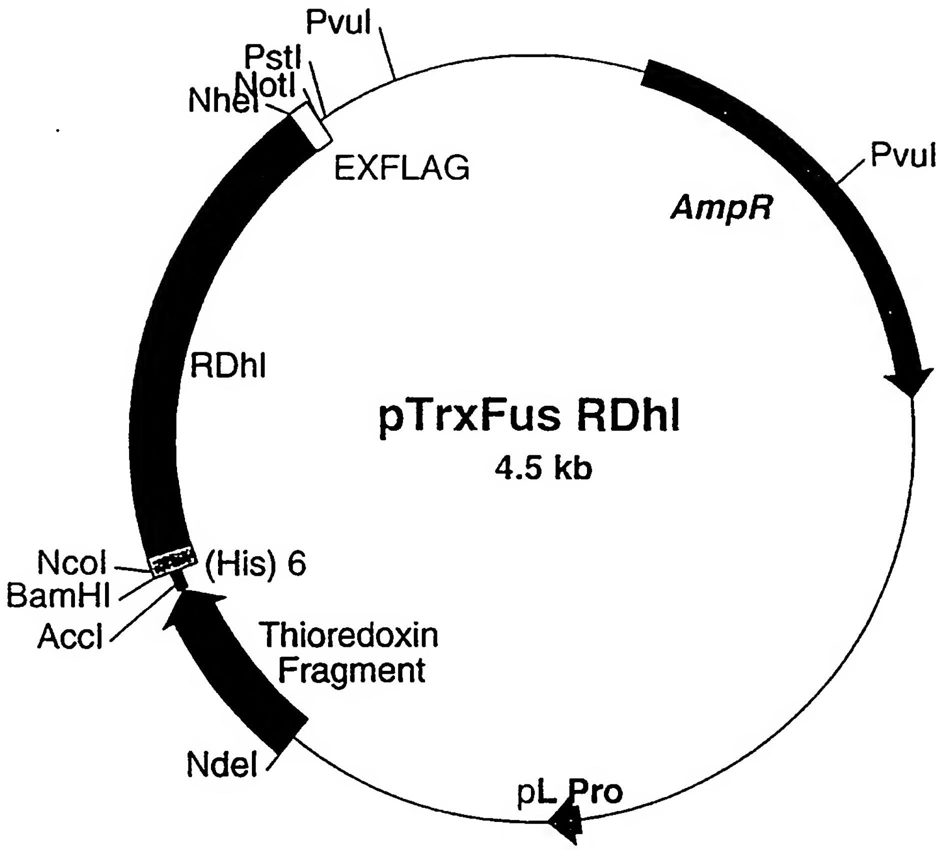
Fig. 7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BamHI
AccI
(His) 6
Thioredoxin Fragment
NdeI
pL Pro

Fig. 8

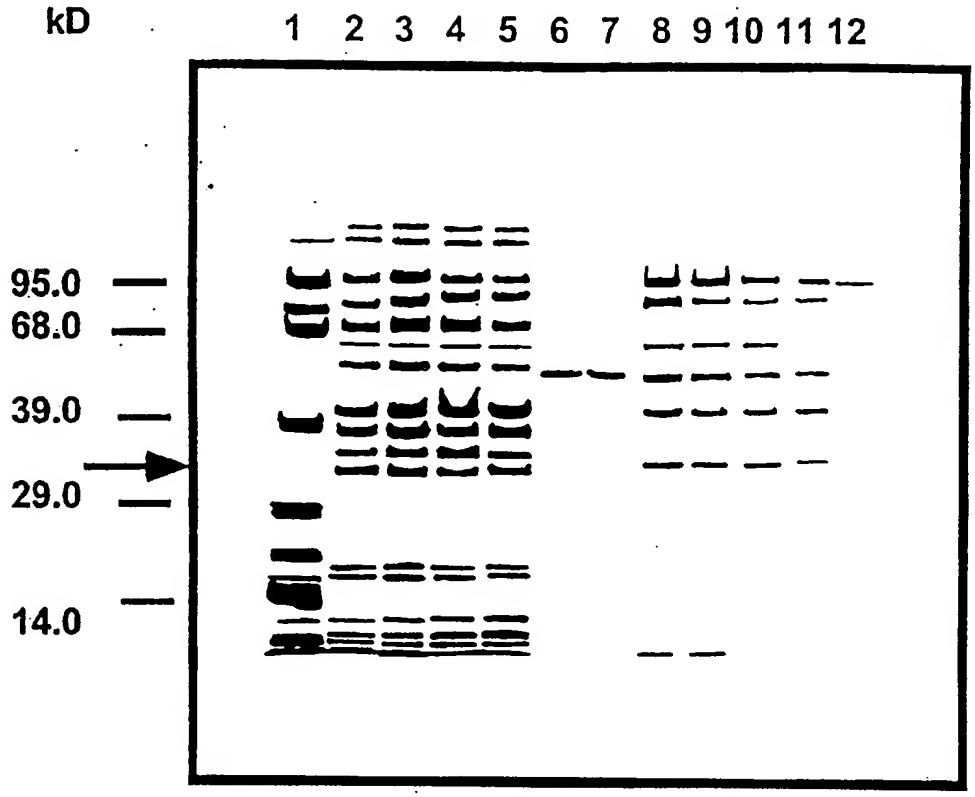

Fig. 9

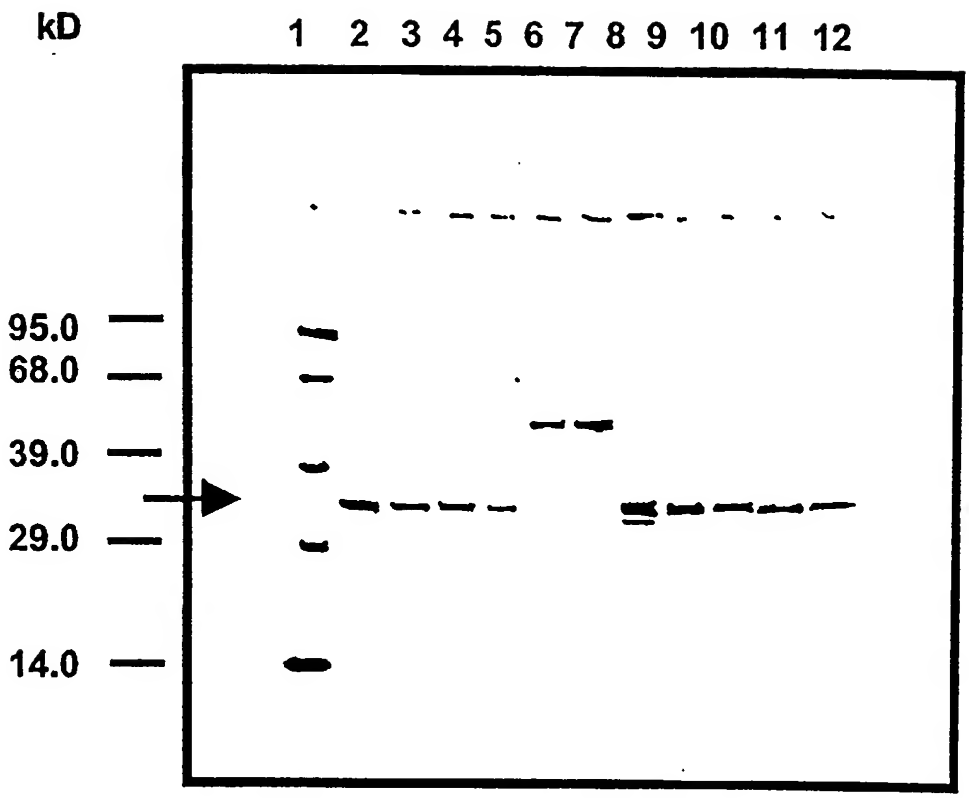

Fig. 10

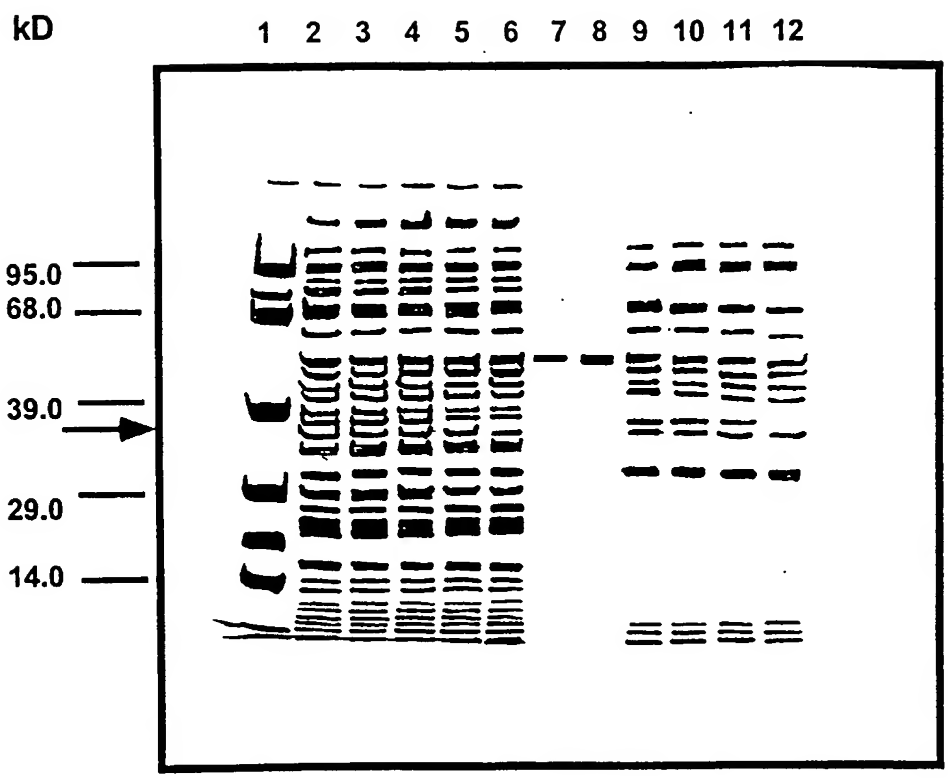

Fig. 11

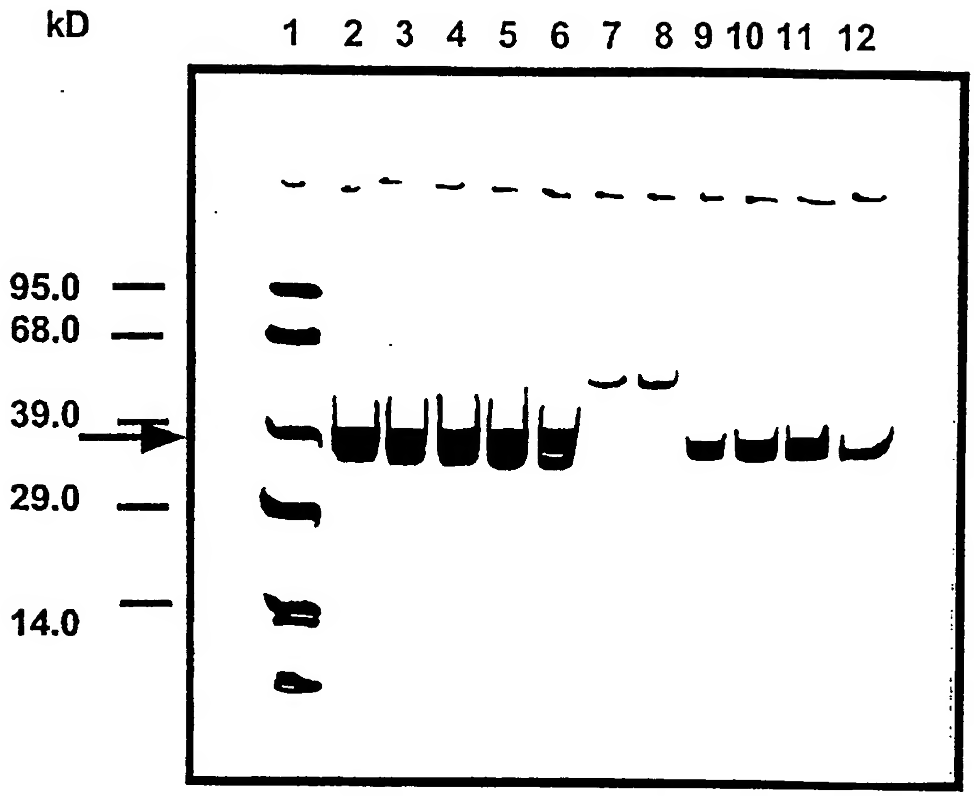

Fig. 12

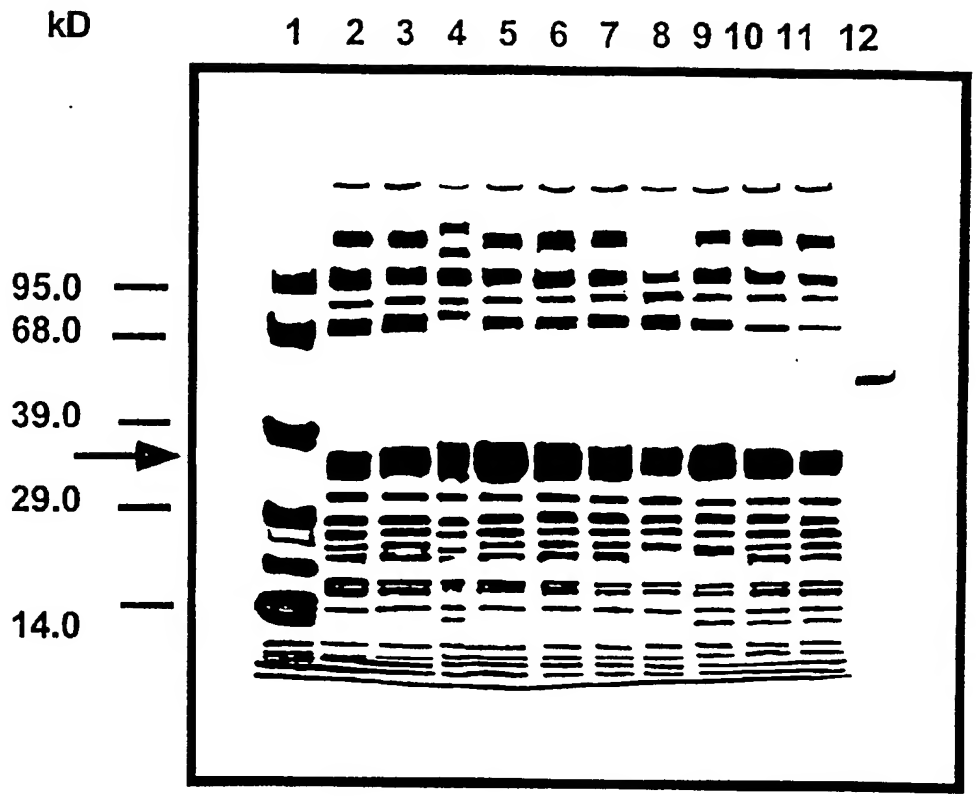

Fig. 13

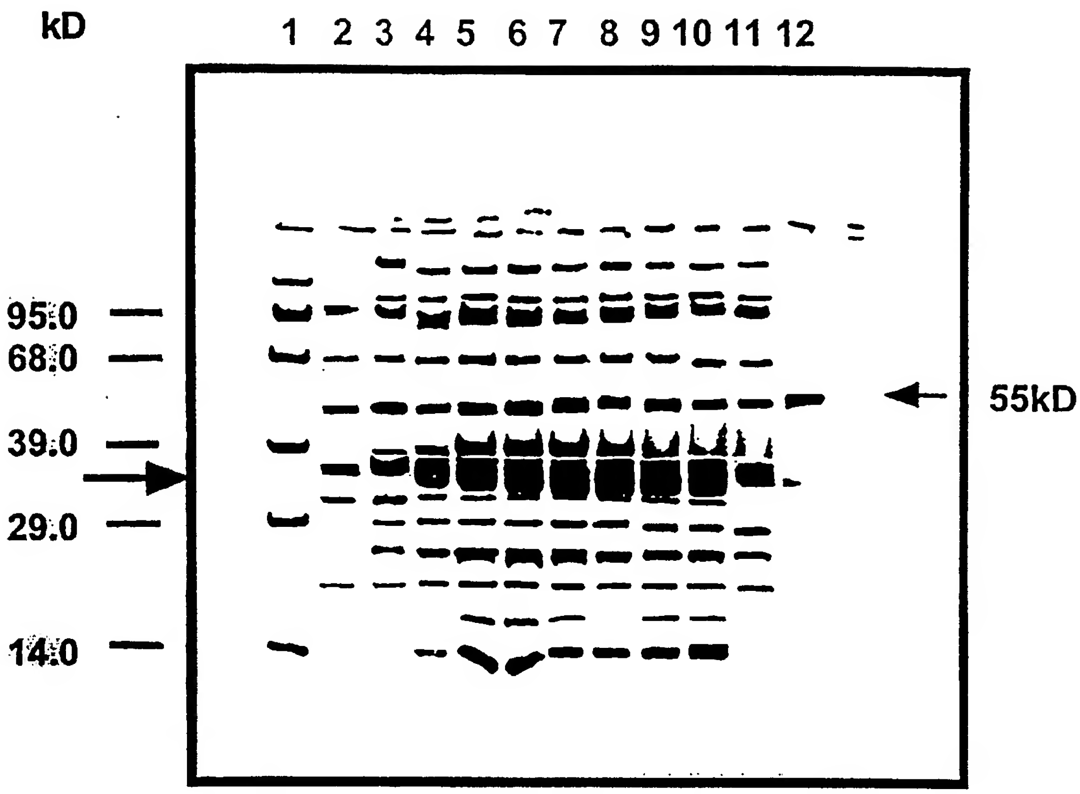

Fig. 14

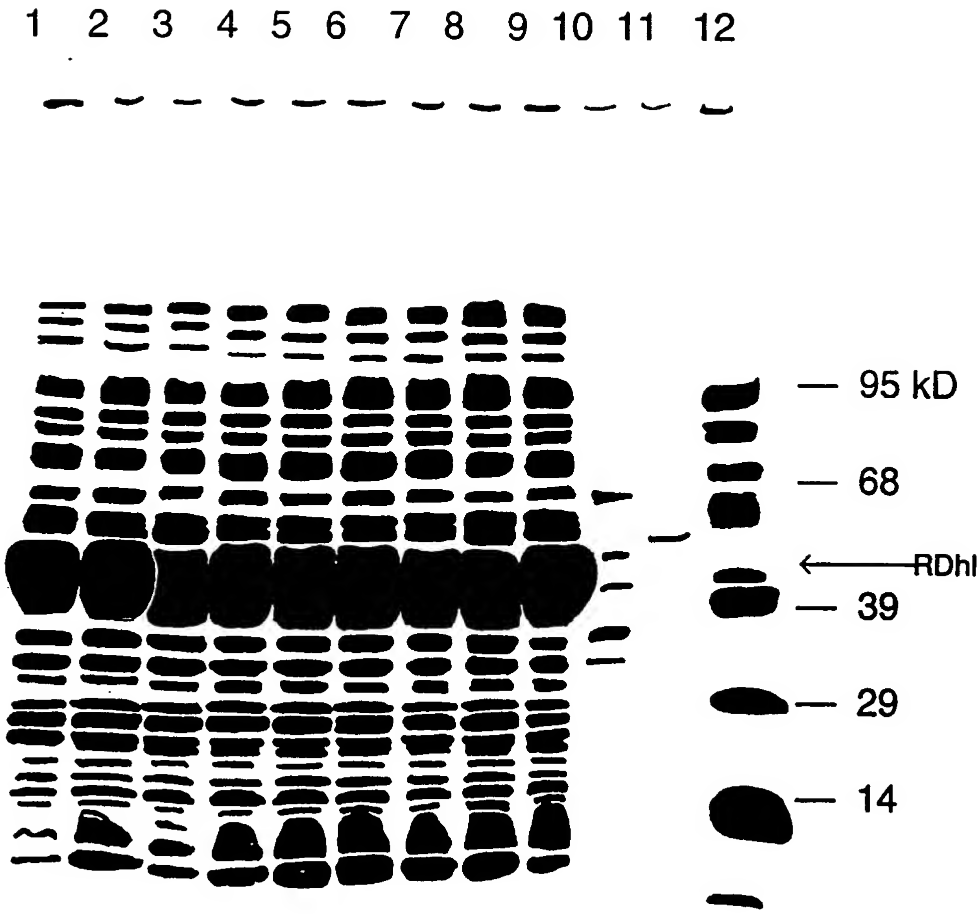

Fig. 15

Fig. 16

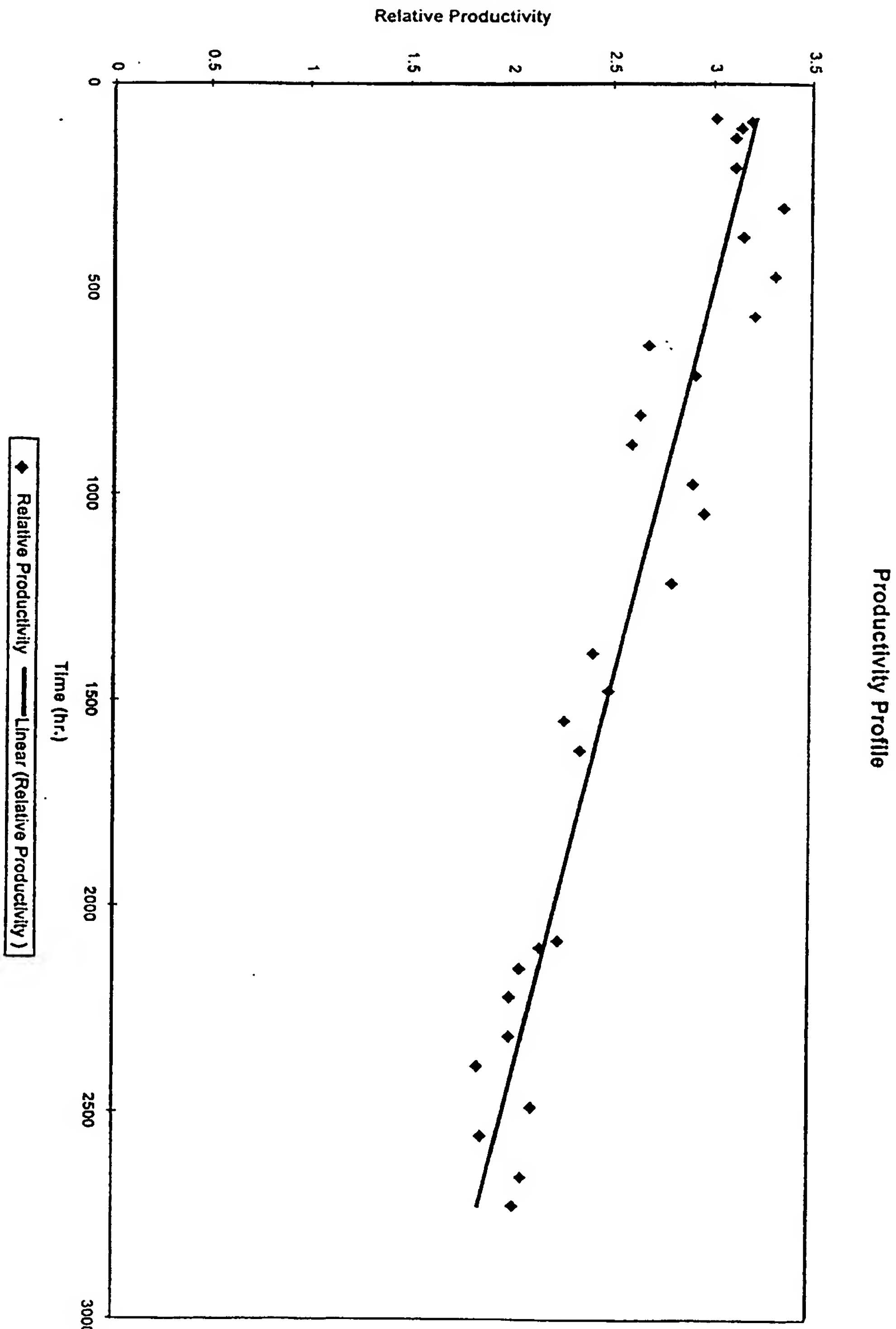
Productivity Profile
Relative Productivity
0
0.5
1
1.5
2
2.5
3
3.5
500
1000
1500
2000
2500
3000
Time (hr.)
Relative Productivity
Linear (Relative Productivity)

Fig. 17. Activity of EPPCR-Mutated RDhl Enzymes

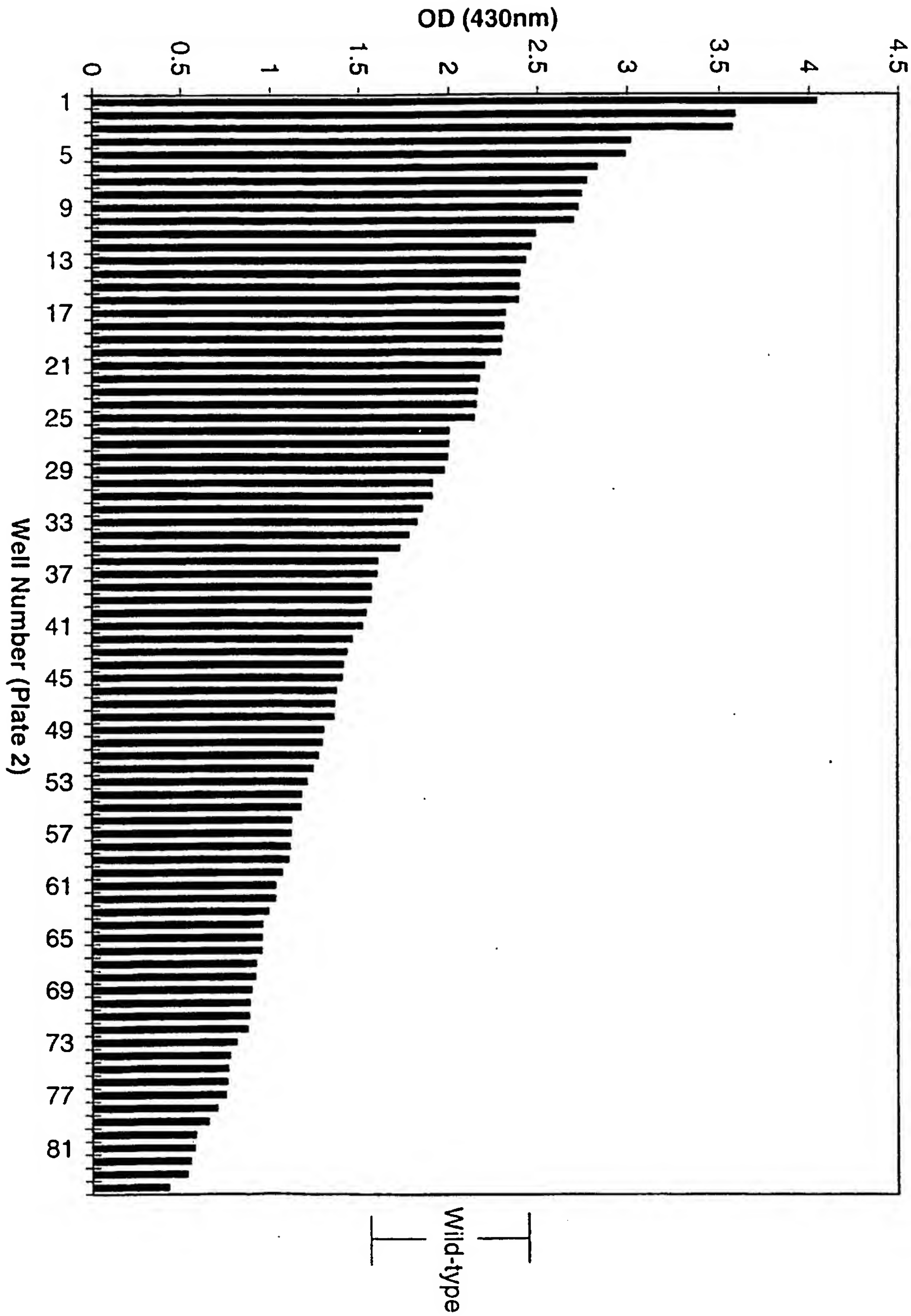

Fig. 18. Activity of EPPCR-Mutated RDhl Enzymes

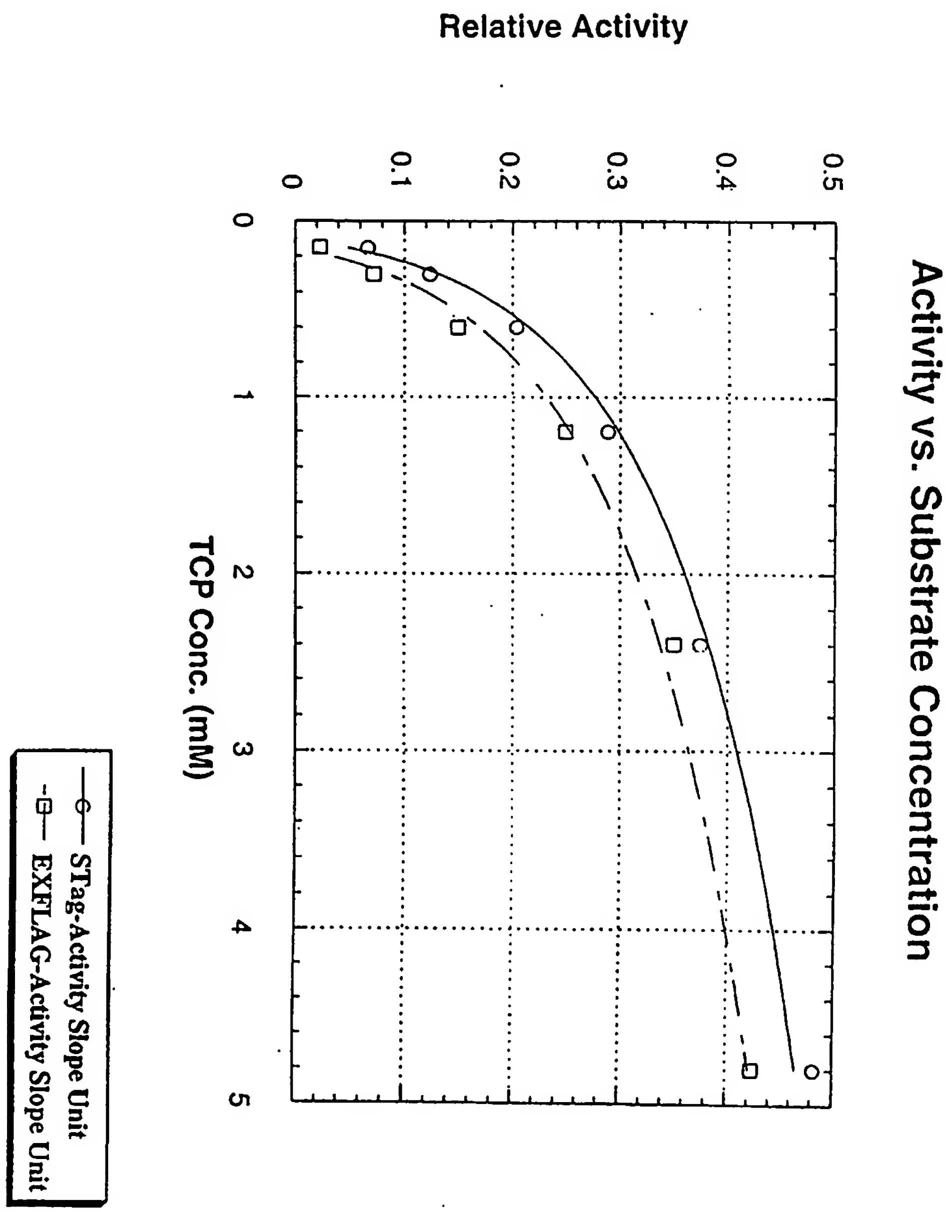
Activity vs. Substrate Concentration
Relative Activity
0
0.1
0.2
0.3
0.4
0.5
0
1
2
3
4
5
TCP Conc. (mM)
STag-Activity Slope Unit
EXFLAG-Activity Slope Unit

Fig. 19

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 98/02776

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N9/14 C12N1/21 C12N1/15 C12N11/00
C12N15/70 //(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KULAKOVA, ANNA N. ET AL: "The plasmid-located haloalkane dehalogenase gene from Rhodococcus rhodochrous NCIMB 13064" MICROBIOLOGY (READING, U. K.) (1997), 143(1), 109-115 CODEN: MROBEO;ISSN: 1350-0872, XP002070886 see the whole document	1-13
X	& DATABASE EMBL Entry RRDHLA 19 FEB 1996 AC L49435 KULAKOVA ET AL. Rhodococcus rhodochrous 1-chloroalkane halidohydrolase (dhlA) gene	4-13

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1998	17.07.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Delanghe, L

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL Entry Q53042 01 nov 1996 AC Q53042 KULAKOVA A.N. ET AL. 1-CHLOROALKANE HALIDOHYDROLASE XP002070888 see the whole document ---	1-3
A	EP 0 657 531 A (DOW CHEMICAL CO) 14 June 1995 see the whole document & US 5 372 944 A (PAUL E. SWANSON) 12 December 1994 cited in the application see the whole document ---	1-3
A	CHEMICAL ABSTRACTS, vol. 123, no. 17, 23 October 1995 Columbus, Ohio, US; abstract no. 222412, ARMFIELD, SUSAN J. ET AL: "Dehalogenation of haloalkanes by Rhodococcus erythropolis Y2. The presence of an oxygenase-type dehalogenase activity complements that of an halidohydrolase activity" XP002070889 see abstract & BIODEGRADATION (1995), VOLUME DATE 1995, 6(3), 237-46 CODEN: BIODEG;ISSN: 0923-9820, ---	1
X	EP 0 276 560 A (IMPERIAL CHEMICAL INDUSTRIES PLC, UK) 3 August 1988 see claims ---	11
X	WO 93 20223 A (ZENECA LTD ;BYROM DAVID (GB); ABBISHAW BARBARA ANN (GB)) 14 October 1993 see claims ---	11
X	CHEMICAL ABSTRACTS, vol. 114, no. 21, 27 May 1991 Columbus, Ohio, US; abstract no. 205473, PARKER, KAREN: "The immobilization of a stereospecific dehalogenase from Pseudomonas putida AJ1/23" XP002070890 see abstract & (1989) 183 PP. AVAIL.: UNIV. MICROFILMS INT., ORDER NO. BRDX90951 FROM: DISS. ABSTR. INT. B 1991, 51(8), 3828-9, 1989, --- -/--	11

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 123, no. 15, 9 October 1995 Columbus, Ohio, US; abstract no. 192321, PARKER, KAREN ET AL: "Immobilization of the D-2-haloacid dehalogenase from Pseudomonas putida strain AJ1/23" XP002070891 see abstract & BIODEGRADATION (1995), VOLUME DATE 1995, 6(3), 191-201 CODEN: BIODEG;ISSN: 0923-9820, 1995, ---	11
X	DIEZ, A. ET AL: "Improved catalytic performance of a 2-haloacid dehalogenase from Azotobacter sp. by ion-exchange immobilization" BIOCHEM. BIOPHYS. RES. COMMUN. (1996), 220(3), 828-33 CODEN: BBRCA9;ISSN: 0006-291X, 1996, XP002070887 see the whole document ---	11
X	CHEMICAL ABSTRACTS, vol. 122, no. 19, 8 May 1995 Columbus, Ohio, US; abstract no. 234875, PLOEG, JAN VAN DER ET AL: "Genetic adaptation of bacteria towards chlorinated hydrocarbon degradation" XP002070927 see abstract & BIOSAFETY RESULTS FIELD TESTS GENET. MODIF. PLANTS MICROORG., PROC. INT. SYMP., 2ND (1992), 163-9. EDITOR(S): CASPAR, RUDOLF;LANDSMANN, JOERG. PUBLISHER: BIOL. BUNDESANST. LAND- FORSTWIRTSCH., BRAUNSCHWEIG, GERMANY. CODEN: 60QTAB, ---	37-39
X	CHEMICAL ABSTRACTS, vol. 119, no. 3, 19 July 1993 Columbus, Ohio, US; abstract no. 23632, ASMARA, WIDYA ET AL: "Protein engineering of the 2-haloacid halidohydrolase IVa from Pseudomonas cepacia MBA4" XP002070928 see abstract & BIOCHEM. J. (1993), 292(1), 69-74 CODEN: BIJOAK;ISSN: 0306-3275, -----	37,38

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Inte. .ational application No.
PCT/US 98/02776

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-13,23-36,44,45

   ENZYME WITH DEHALOGENASE ACTIVITY AND SEQUENCE OF FIGURE 2, FUSION PROTEINS,DNA,EXPRESSION SYSTEM,HOST AND IMMOBILIZATION ON A CARRIER

2. Claims: 14-22

   AN IMMOBILIZED ENZYME HAVING A HALOALKANE DEHALOGENASE ACTIVITY WITHOUT SPECIFIED SEQUENCE

3. Claims: 37-43

   AN ENZYME WITH DEHALOGENASE ACTIVITY,WHOSE DNA HAS BEEN DERIVED,BY A DIRECTED EVOLUTION PROCESS,FROM A RELATED DNA SEQUENCE

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/02776

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0657531 A	14-06-1995	US 5372944 A	13-12-1994
		BR 9403715 A	30-05-1995
EP 0276560 A	03-08-1988	DK 40688 A	28-07-1988
		FI 880130 A	28-07-1988
		JP 4048438 B	06-08-1992
		JP 63202383 A	22-08-1988
		US 4968605 A	06-11-1990
WO 9320223 A	14-10-1993	AU 3894593 A	08-11-1993
		DE 69315570 D	15-01-1998
		EP 0632837 A	11-01-1995
		JP 7508161 T	14-09-1995
		US 5519130 A	21-05-1996